Use of Zonal Ultracentrifuge Systems for Biophysical Studies of Rhinoviruses

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This paper reports the use of zonal ultracentrifuge techniques to conduct biophysical studies of rhinoviruses grown with WI-38 cells. Good clean-out of infectivity from rhinovirus harvests was obtained with the continuous-flow B-V and B-IX rotors. Use of the B-V rotor resulted in the successful concentration of rhinovirus infectivity and antigenicity. Additional purification was achieved by the combined use of continuous-flow centrifugation and isopycnic banding procedures. Two particle sizes were found to be associated with the virus-infected cell harvests. The infectious 22-nm particle banded in density ranges of 1.38 to 1.40 g/cm³ in CsCl and 1.26 to 1.27 g/cm³ in potassium citrate. The 8.0 nm capsomere was composed of 2.0 nm subunits and banded with a density of protein at 1.28 g/cm³ in CsCl. Equivalent sedimentation coefficients of 155 or 185, depending on particle density in sucrose, were calculated from rate zonal experiments by use of the B-IV zonal rotor.

Rhinoviruses are associated with upper respiratory illnesses in adults. They are classified as a subgroup of the picornaviruses and are distinguished from the enterovirus subgroup by their acid lability (16).

In view of the large number of known rhinovirus serotypes (11) and their poor growth in tissue culture, methods for the concentration and purification of rhinoviruses were considered for the purpose of producing concentrated polyvalent rhinovirus vaccines. Recent developments in zonal centrifuge technology, described by Anderson and his associates at the Oak Ridge National Laboratories (1, 3), seemed applicable for this purpose. The use of these centrifuge systems for the biophysical characterization of rhinoviruses is reported in this paper.

METHODS AND MATERIALS

Rhinoviruses. The rhinovirus numbering system is that recommended by the Directors of the World Health Organization Respiratory and Enterovirus Reference Center (15). Rhinovirus strains 1A, 1B, 2,

13, 14, and 17 were used in this study. Their history was given in an earlier report (10).

Tissue culture, virus growth, and bioassays. The human embryonic diploid WI-38 strain used in these studies was obtained from Flow Laboratories, Rockville, Md. and from L. Hayflick of the Wistar Institute in Philadelphia, Pa. The methods used for the passage of WI cells were as described by Hayflick and Moorhead (12). Conditions of cell growth were given in an earlier report (10).

WI-38 cell cultures were infected with approximately 10² TCID₅₀ of rhinovirus, and harvested when 80 to 100% of the cells showed cytopathic effects (13). Harvests were pooled and held at -20 C. Infectivity titrations and serum neutralization tests were done in tissue culture tubes of WI-38 cells and rolled at 30 to 32 C according to published procedures (10).

Preparation of guinea pig antiserum. Rhinovirus harvest or B-V concentrate was injected into guinea pigs by either of two protocols. After a preimmunization bleeding, a group of pigs was injected intraperitoneally with 2 ml of aqueous virus twice a week for 2 weeks (protocol A), or intramuscularly with two 1-ml injections of aqueous virus 3 weeks apart (protocol B). All guinea pigs were bled 1 week after the last injection.

Continuous-flow centrifugation in the B-V rotor. Barringer et al. (3) have described the B-V rotor and its operation. Rhinovirus harvest material was processed in the B-V rotor at 40,000 rev/min at 5 C at various flow rates under aseptic conditions. After processing, the rotor was braked to a stop and the pelleted material was resuspended in the rotor contents. Pellet resuspension was aided by the use of a rubber policeman or by gentle shaking of the rotor at

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4 C overnight before unloading, or both. The suspended pellet (130 ml) and the final rinse [20 ml of 0.01 M phosphate-buffered saline (PBS)] were then suctioned directly into a sterile vacuum bottle.

Combined continuous-flow centrifugation and isopycnic banding in the B-IX rotor. Anderson et al. (1) have described the B-IX rotor and its typical operation. The B-IX rotor was loaded while spinning at 5,000 rev/min with either a 750-ml two-step (1.1 and 1.4 g/cm³) potassium citrate gradient or a programmed 600-ml linear (1.1 to 1.5 g/cm³) potassium citrate gradient followed by a 150-ml 1.5 g/cm³ potassium citrate cushion. The rotor was accelerated to speed and the harvest was processed through the rotor at various flow rates. When the starting sample was expended, the rotor was run for 1 to 2 hr to ensure equilibrium and then was decelerated without braking to 5,000 rev/min. The density gradient was unloaded by pumping dense potassium citrate to the rotor periphery and the resulting effluent was collected in thirty 25-ml fractions.

Rate separation in the B-IV rotor. The B-IV rotor (2) was loaded at 5,000 rev/min with a linear 1,200-ml 10 to 55% (w/w) sucrose gradient followed by a 500-ml (w/w) sucrose cushion by use of a Beckman (model 131) high capacity gradient pump. Sterile sucrose solutions were made by dissolving sucrose crystals in 0.01 M PBS (0.85%) and purified in our laboratory by passage over granular charcoal columns. The rhinovirus sample was introduced into the rotor in a 25-ml volume followed by a 200-ml overlay of PBS, displacing an equivalent volume of cushion from the rotor periphery. The rotor was accelerated to 40,000 rev/min, run for 90 min at 5 C, and decelerated without braking to 5,000 rev/min. The gradient was recovered from the rotor by displacement with 55% (w/w) sucrose solution and collected in 85 fractions of 20 ml each.

Sedimentation coefficients were calculated (courtesy of N. G. Anderson) by the computer program at the Oak Ridge National Laboratories. Equivalent sedimentation coefficients (S^* ; 4) were returned corresponding to the center of each fraction for a range of particle densities from 1.2 to 1.6 g/cm³.

Isopycnic banding in cesium chloride density gradients. Rhinoviruses were banded in linear preformed cesium chloride gradients (1.1 to 1.6 g/cm³) in the SW 25.1 rotor at 25,000 rev/min for 20 hr at 5 C. By unloading the centrifuge tubes from the bottom, the gradients were recovered and collected in 2-ml fractions.

Density measurements. Potassium citrate gradients were either determined by the use of standard curves relating density to refractive index or measured directly with a pycnometer. Refractive indices were read on the Abbe 3L refractometer for the determination of cesium chloride densities and sucrose concentrations.

Protein. Samples of recovered gradient fractions were dialyzed extensively against PBS at 4 C and assayed for protein concentration by the method of Lowry et al. (17).

Electron microscopy. Dialyzed samples of rhinovirus preparations were negatively stained with 2% phosphotungstic acid (PTA) at pH 7.0 or with saturated aqueous uranyl acetate by use of carbon coated collodion grids. Micrographs were taken with the RCA-EMU-3G microscope at initial magnifications of 50,000 $\times$ or 100,000 $\times$.

RESULTS

Concentration of rhinoviruses by direct pelleting in the B-V rotor. Figure 1 shows the results of clean-out studies with rhinovirus 1A in the B-V continuous-flow rotor. More than 95% of the infectivity was removed at flow rates up to 4 liters per hr. Table 1 shows the concentration of rhinovirus infectivity in the resuspended contents of the B-V rotor for a number of separate experiments in which rhinovirus 1A or 13 harvests were used. Table 2 shows the concentration of rhinovirus antigenicity, as measured by guinea pig potency tests, for several experiments in which either live or Formalin-inactivated harvests were used as starting material. Although complete recovery of infectivity was not always obtained, good concentration of antigenicity was achieved in all cases. An average 30-fold purification, on a protein basis, was also obtained by this direct pelleting procedure. We have concentrated up to 50 liters of membrane-filtered (0.45 μ , Millipore Corp., Bedford, Mass.) rhinovirus harvest in a single B-V run under continuous operation.

Examination of the B-V concentrates by electron microscopy revealed the presence of 22-nm virus like particles and numerous small annular

FIG. 1. Three experiments with rhinovirus 1A harvests showing the clean-out of infectivity at 40,000 rev/min as a function of flow rate in the B-V rotor.

TABLE 1. *Clean-out and concentration of rhinovirus infectivity using the B-V rotor*

Expt no.	Virus type	Flow ^a rate	Per cent clean-out ^b	Concn by vol	Concn by ^c TCID ₅₀ /ml
1	1A	0.8	98	11×	10×
2	1A	1.0	99	16×	10×
3	13	1.0	97	18×	5×
4	13	0.8	99.9	22×	10×
5	13	1.7	>99	35×	32×
6	13	2.0	>99	18×	3×
7	13	2.4	No test	33×	32×
8	13	2.4	No test	33×	32×

^a Flow rate is expressed in liters per hour.^b The removal of rhinovirus from harvest, as calculated from difference in infectivity titer between harvest and final effluent.^c Concentration, as calculated from difference in infectivity titer between starting sample and B-V concentrate.TABLE 2. *Concentration of rhinovirus antigenicity using the B-V continuous-flow rotor*

Expt no.	Virus type	Concn by vol	Protocol ^a	Guinea pig potency endpoint ^b	
				Harvest	Concentrate
1	1A	16×	A	1:5	1:100
2	13	33×	A	1:5	1:125
3 ^c	13	200×	A	1:5	1:50
4 ^c	14	78×	B	1:5	1:625
5 ^c	14	80×			

^a Guinea pig injection schedule as described in Methods and Materials.^b Determined by geometric mean of serum neutralization titer.^c Harvest membranefiltered (0.45 μ) and Formalin inactivated prior to processing in the B-V rotor. In experiment 3, the 200 $\times$ concentrate was diluted to 20 $\times$ with medium 199. In experiments 4 and 5, the concentrates were pooled and then diluted to 30 $\times$ with medium 199. The concentrates at this final dilution were then injected according to stated protocol.

structures, associated with an appreciable amount of fine debris. Figure 2A shows a 35 $\times$ concentrate of a rhinovirus 1A harvest. Twenty-five virus particles were measured and found to be 21 to 23 nm in diameter. When viewed at lower magnification, most appeared to have hexagonal outlines, and higher magnification revealed the presence of capsomeres (Fig. 2B). Occasional "empty" or partly empty particles were observed in which the stain had penetrated the core and left a ring of

capsomeres at the periphery (Fig. 2C). The preparations stained by the uranyl acetate technique were much more illuminating than those prepared with PTA; the capsomeres were more distinct. The hexagonal outline and the presence of capsomeres suggested an icosahedral structure.

The annular structures or "donuts" measured 8.0 to 9.0 nm in diameter with a 2.5 nm hollow core, and were composed of subunits 2.0 to 3.0 nm in diameter (Fig. 2D). In well-oriented particles, there were five or six subunits per capsomere, although exact counts often were difficult. The best counts were made on reversed prints as shown by Fig. 2E. Neither the 22-nm particle nor the small annular donut was observed in uninfected cell controls.

Isopycnic banding of rhinovirus B-V concentrates in CsCl density gradients. When the rhinovirus concentrate shown in Fig. 2A was banded in a cesium chloride density gradient, the infectivity was fully recovered at a density of 1.38 g/cm³. Figure 3 shows the distribution of infectivity on the recovered gradient. Examination of dialyzed fractions by electron microscopy revealed that the 22-nm particle was associated only with the infectivity peak and was well separated from protein impurities. The 8.0 nm donut, however, was found at a density of 1.28 g/cm³, and was well separated from the peak infectivity.

We have banded repeatedly a number of rhinovirus types in density gradients, and all exhibited this high density in cesium chloride. Rhinovirus types 1A, 1B, 2, 13, 14, and 17 all banded sharply in a range from 1.38 to 1.41 g/cm³, with good recovery of infectivity.

Continuous-flow centrifugation and isopycnic banding of rhinovirus in the B-IX zonal rotor. Clean-out studies with the B-IX rotor were done, and Fig. 4 shows the removal of rhinovirus infectivity, at various flow rates, at 40,000 rev/min. Again, as with the B-V rotor, good clean-out of infectivity was achieved at flow rates up to 4 liters per hr. Figure 5 shows the distribution of infectivity in a potassium citrate density gradient after processing 3 liters of rhinovirus 1A harvest at 30,000 rev/min at an average flow rate of 1.3 liters per hr. The results are expressed as per cent of recovered infectivity in order to better represent the mass relationships. Although only 30% of the starting infectivity was recovered on the gradient, the infectivity peaked sharply at a density of 1.262 g/cm³. Examination of the peak infectivity fraction by electron microscopy revealed the presence of the 22-nm particle in good concentration. Only fine protein material was found in the background, which demonstrated that better purification was achieved through use of the B-IX rotor than by direct pelleting. In another

FIG. 2. Electron micrographs of a rhinovirus 1A B-V concentrate. (A) Viral particles and individual capsomeres. $\times 175,000$. (B) An intact viral particle stained with uranyl acetate. $\times 400,000$. (C) A viral particle in which stain has penetrated the core. $\times 400,000$. (D) Individual capsomeres, negatively stained with uranyl acetate. $\times 500,000$. (E) Reverse contrast print of a single isolated capsomere illustrating subunits. $\times 500,000$.

experiment of this type with rhinovirus 13, the peak infectivity banded at 1.265 g/cm^3 in potassium citrate, and, when the virus at this density was rebanded in a cesium chloride gradient, the 22-nm particle was recovered at a density of 1.40 g/cm^3 . The distribution of rhinovirus infectivity in potassium tartrate was similar to that observed with potassium citrate gradients. The marked difference between the buoyant density values in potassium citrate and in cesium chloride reflects the solvation properties of the rhinovirus particle.

Rate separation of rhinovirus in the B-IV zonal

rotor. Figure 6 shows the results of a rate zonal run in the B-IV rotor in a sucrose density gradient. The starting sample was the peak infectivity fraction recovered from an isopycnic run in potassium citrate, such as that shown by Fig. 5, dialyzed against PBS at 4°C and concentrated by pelleting at 30,000 rev/min in the Spinco 30 rotor. Again, the results are expressed as per cent of recovered activity, and full recovery of the starting infectivity was obtained. The infectivity peak moved as a homogeneous band with possibly a slight lead zone. Good additional purification was obtained

by this second-step rate zonal procedure. Sedimentation coefficients were calculated by digital computation at the Oak Ridge National Laboratories. Assuming a particle density of 1.40 g/cm^3 ,

FIG. 3. Distribution of rhinovirus 1A infectivity in cesium chloride density gradient after banding for 20 hr at 25,000 rev/min and 4 C in the SW 25.1 rotor.

FIG. 4. Clean-out of infectivity from rhinovirus 13 harvest in the B-IX rotor at 40,000 rev/min as a function of flow rate. Rotor was preloaded with a potassium citrate density gradient.

FIG. 5. Combined continuous flow centrifugation and isopycnic banding in the B-IX rotor. Distribution of rhinovirus infectivity in a potassium citrate gradient.

FIG. 6. Rate separation of rhinovirus using a sucrose gradient in the B-IV rotor.

as found in cesium chloride, the S^* was 155. If the buoyant density value for the peak infectivity obtained in potassium citrate is used, the S^* is 185. The particle density of rhinovirus in sucrose is not known.

The 22-nm virus particle could be separated from the 80 A donut by this rate procedure in sucrose, as well as by the isopycnic cesium chloride method mentioned previously.

DISCUSSION

Rhinoviruses are among the smallest of the known viruses, and they represent quite a chal-

lenge to continuous-flow centrifugal techniques. Yet, good clean-out and concentration of these viruses at flow rates up to 4 liters per hr were obtained in these studies with the zonal centrifuge systems. Reimer et al. (22) reported similar success in the use of the B-V rotor with poliovirus. We have used the B-V rotor for the concentration of two monovalent rhinovirus vaccines that are scheduled for clinical studies. The combined continuous-flow centrifugation and isopycnic banding technique, followed by rate separation ($\rho - S$), was applicable for the concentration and purification of rhinoviruses.

The average diameter of the rhinovirus particle was 22 nm, which agrees with published values. Mayor (18) reported a diameter of 20 to 24 nm for rhinovirus 45, and Chapple and Harris (6) published a diameter of 30 nm for rhinovirus 2. Ultrafiltration studies placed the particle size in the range of 15 to 30 nm (21). The 8.0 nm annular structure, or donut, was found associated only with virus-infected cells, and it could represent the viral capsomere. Capsomeres of this size and shape were observed with papilloma and polyoma viruses (14) and with foot-and-mouth disease virus (23). The donut had a buoyant density of protein at 1.28 g/cm³ in cesium chloride. We have not been able to obtain a sufficient quantity of this material in a purified state to determine the antigenic relationship to the infectious rhinovirus particle.

The high density in cesium chloride for a number of rhinoviruses was reported by Dans (7), McGregor (20), and Chapple (6). In our studies, six rhinovirus types showed this same behavior and, considering all published reports, all of seven types selected had this property in common. The enteroviruses do not share this property. This characteristic could be considered as a basis for the classification of this subgroup of the picornaviruses. Actually, the high density in cesium chloride and the known acid lability of these viruses probably are different expressions of the same physical property.

Rhinoviruses have a higher density in cesium chloride (salt of a heavy cation) than in either potassium citrate or potassium tartrate (salts of heavy anions). This marked solvation effect might be due to binding of cesium ions to negatively charged viral ribonucleic acid (RNA), which is somehow more accessible for the rhinoviruses than for the enteroviruses. The acid lability of the rhinoviruses would seem to support this possibility. McGregor and Mayor (19) suggested recently that the increased density of rhinoviruses over the acid-insensitive picornaviruses was due to an increased RNA content.

The sedimentation coefficient values obtained

in these studies for rhinoviruses were somewhat higher than those reported (5, 24) for foot-and-mouth disease virus (140S) or poliovirus (154S). However, the solvation properties of rhinovirus in sucrose are not known. Dimmock and Tyrrell (8) reported sedimentation coefficients of 165 for rhinovirus 1B and 155 for rhinovirus 2.

Much larger quantities of the virus are required for further biochemical and biophysical analyses than are obtainable. A recent report on the propagation of rhinoviruses to higher titers in rolling bottles (9) offers promise in this direction.

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