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Respiratory Syncytial Virus Isolation by Combined Continuous Flow-Isopycnic Banding Centrifugation

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A new zonal centrifuge rotor (B-IX) which combines continuous sample flow centrifugation with isopycnic banding has been used to isolate and concentrate respiratory syncytial virus from liter volumes of culture fluid. This isolation technique utilizes a sucrose density gradient to trap and isopycnically band the virus particles, and permits recovery of the particles from the rotor in an unaggregated condition.

The development of a new continuous-flow rotor, the B-IX system, for the zonal centrifuge has made it possible to isolate, concentrate, and recover infectious viruses which are inactivated by conventional pelleting by centrifugation procedures (1). This report describes the application of the new rotor system for the isolation of respiratory syncytial (RS) virus from liter volumes of infected tissue culture fluid.

RS virus, a major cause of severe respiratory disease in infants and young children (7), has been difficult to study because of the relatively low yield of virus and viral antigens from infected cell cultures. Although the infectious particle can be sedimented by high-speed centrifugation, conventional centrifugation by pelleting has proven of limited value as a means of concentrating the virus, since 80 to 90% of the infectivity can be lost during sedimentation (5). However, in a recent study, Forsyth, Coates, and Chanock (8) demonstrated that RS virus and one of its associated complement-fixing (CF) antigens can be concentrated by equilibrium sedimentation in cesium chloride.

The HEp-2 grown RS virus used in this study is considered merely as a test particle. It is hoped that the behavioral characteristics of this particle in this isolation technique can be used to advantage in future preparation of potentially useful vaccine materials.

MATERIALS AND METHODS

Virus. The Long strain of RS virus, which had been transferred 12 to 14 times in human continuous cell lines (K 13, Chang liver, HEp), was used for all studies (5). Large pools of infected tissue culture fluids were prepared in HEp-2 cell monolayers grown in 32-oz (0.95-liter) prescription bottles or Povitsky bottles, as described by Forsyth, Coates, and Chanock (8). The tissue culture harvest was clarified to remove large cellular debris by centrifugation at 2,000 rev/min for 20 min in a PR 2 International refrigerated centrifuge. After clarification, the fluids were either shell frozen in an alcohol-dry ice bath and stored at −70 C, or were held at 4 C until used (24 to 48 hr).

Bioassays. Virus assay was carried out by plaque titration in HEp-2 cell monolayers as described by Coates, Alling, and Chanock (6). Titers were calculated from the geometric mean number of plaques and expressed as plaque-forming units (PFU) per milliliter. The standard error of such titration was estimated by dividing the range of counts at a specific dilution by the number of replicate plates counted. In no case did the estimated standard error exceed $10^{0.3}$.

Chanock et al. (4) previously described the method used to measure CF antigen in a micro CF test. Antigen titrations were carried out with 4 units of a human convalescent serum obtained after experimental infection of an adult male with 11657 strain of RS virus (9). This serum contained CF antibody reactive with both antigen A and antigen B of RS virus described by Forsyth, Coates, and Chanock (8). CF antigen titers are expressed in complement-fixing units (CFU) per 0.025 ml, and represent the reciprocal of the highest dilution giving at least a 75% fixation of complement.

The zonal centrifuge and the B-IX and B-V rotors are described elsewhere (1–3). Flow-through rates of 4 liters/hr at 40,000 rev/min or 3 liters/hr at 30,000 rev/min rotor speed were used except where noted. All runs except where indicated were made at 3 to 6 C. The density gradient material was sucrose containing 0.9% NaCl and 0.01 or 0.1 M phosphate buffered to *p*H 7.0 to 7.2.

[1] Operated for the U.S. Atomic Energy Commission by the Nuclear Division of Union Carbide Corp.

The gradient containing the virus was unloaded from the rotor at the end of each run by displacement with a denser solution of sucrose, and then was passed through a quartz flow cell while being monitored constantly for absorbancy of the fractions at 260 or 280 mμ. The gradient was collected in 20- or 25-ml fractions, and samples of each fraction were assayed for infectivity and CF antigen. Additional samples of each fraction were dialyzed against phosphate-buffered saline (PBS) and later examined in an electron microscope after negative (phosphotungstate) staining. The results of electron microscopy will be presented elsewhere.

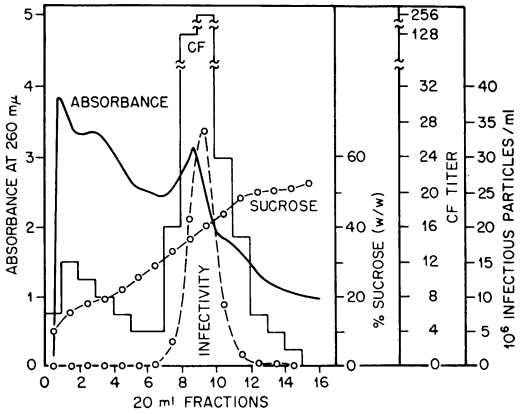

FIG. 1. *Summary of B-IX RS isolation. Total volume, 2.45 liters of previously frozen harvest fluid. Clean-out of infectious virus was 99.75% (estimate based on effluent titer and recovery). Rotor speed was 30,000 rev/min with a flow-through rate of 3 liters/hr.*

The effluent from the B-IX continuous flow rotor was passed through another continuous flow rotor (B-V) at flow rates calculated to collect particles with sedimentation coefficients well below those of the infectious RS virus. The particles which sedimented to the rotor wall were collected after completion of the run. The pellet was suspended in the harvest fluid remaining in the rotor and then neutralized to about *p*H 6.8 with HCl. The suspended sediment from the B-V rotor was concentrated by lyophilization in some experiments to obtain sufficient material to give detectable Schlieren peaks when examined in an analytical ultracentrifuge. Either the standard counterbalance or a wedge cell containing PBS was used in the opposing rotor position. All measurements were made at 20 C. The sedimentation coefficients were calculated by the technique described by Schachman (11) and were uncorrected for ionic effects and concentration of the particles. Assays of total protein were carried out by use of the Miller and Houghton modification of the Kjeldahl nitrogen determination (10). Total protein is defined here as 6.25 times the total nitrogen per milliliter.

RESULTS

Figure 1 shows the results of one experiment in which 2.45 liters of previously frozen harvest fluid containing RS virus (Long strain) was processed in the B-IX rotor. The figure shows the absorbance profile at 260 mμ of the gradient as it was displaced from the spinning rotor. Three main fractions absorbing at 260 mμ are centered in fractions 1, 3, and 9. The absorbance in fraction 1 was associated with the presence of the *p*H indicator which diffused or was sedimented into the gradient. Infectivity assay is shown by the

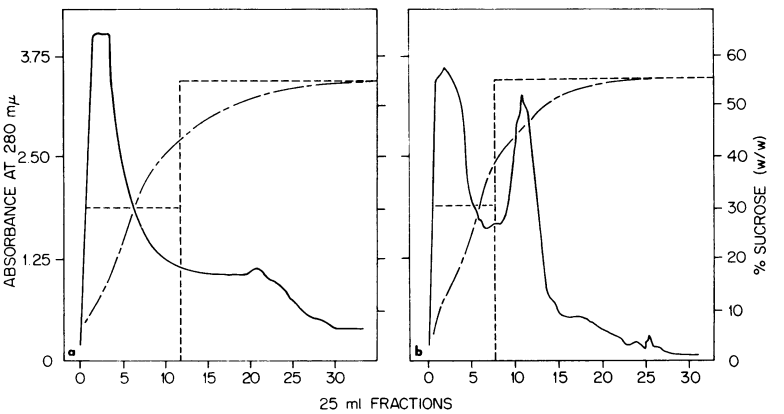

FIG. 2. *Contribution of HEp-2 cellular materials to absorbancy. The solid line is the absorbance at 280 mμ. The dotted line shows the starting sucrose concentrations in the rotor before flow-through was begun. The dot-dash line shows the recovered gradient after the control culture was pumped through the rotors. (a) Absorbance tracing of the sucrose density gradient from the B-IX continuous-flow rotor after about 2 liters of noninfected HEp-2 control culture was pumped through the rotor. Rotor speed was 25,000 rev/min and the flow rate was 2.5 liters/hr. (b) Absorbancy tracing of 3.4 liters of HEp-2 culture infected with virus pumped through at 3.4 liters/hr at a rotor speed of 25,000 rev/min. Infectivity was centered in absorbance peak in fraction no. 11.*

dashed line, and indicates a zone of infectivity centered in fraction 10, which contained 40% sucrose. The distribution of CF antigen is coincident with that of infectivity and its absorbance peak. A smaller peak of CF activity unassociated with infectivity is centered in fraction 3.

As a control, a continuous flow run was made with a noninfected HEp-2 cell culture to determine the contribution of cellular components to the absorbance tracings (Fig. 2a). The dashed line represents the starting sucrose concentrations in the rotor and the dotted line indicates the recovered gradient. Figure 2b shows a comparable run with RS harvest fluid. The peak absorbing at 280 mμ centered in 45% sucrose coincides with the infectivity peak. This peak was not evident in the uninfected HEp-2 control material (Fig. 2a).

Figure 3 shows the absorbance profile, in-

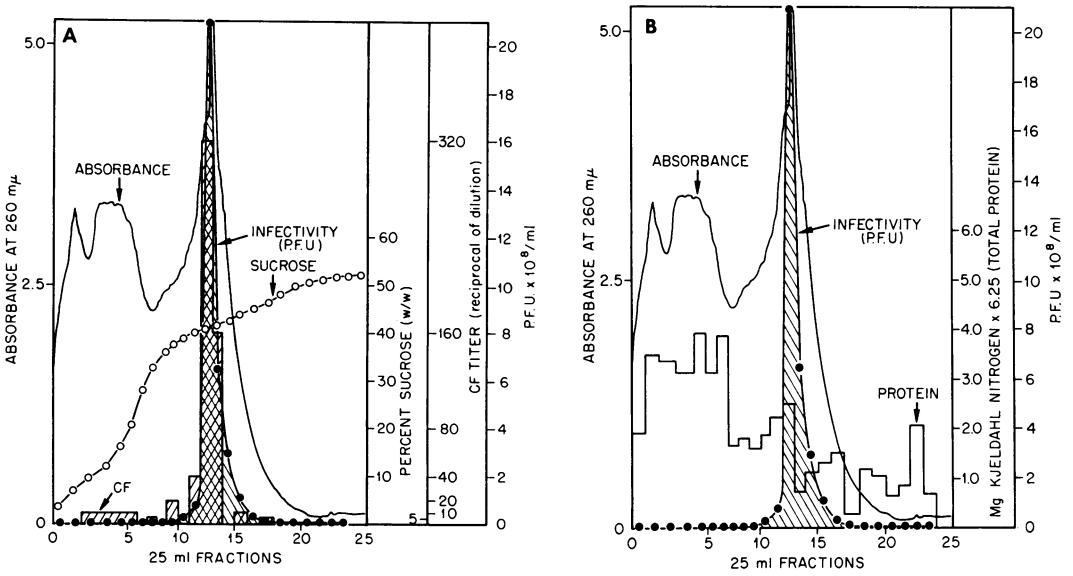

FIG. 3. *Results of a 10-liter flow-through experiment in the B-IX rotor with respiratory syncycial virus harvest fluid. In part A, infectivity (PFU), CF antigen, and sucrose concentrations are indicated for the recovered 25-ml fractions. Part B shows the distribution of protein with respect to both infectivity and absorbance.*

TABLE 1. *Recovery of respiratory syncytial virus by combined continuous flow-isopycnic banding (B-IX rotor)*

Run	Speed	Vol	Flow rate	Banding time after flow-through	Input		Effluent		Infectivity removed from stream	Infectivity recovered in the gradient	Infectivity peak			
					PFU[a]	CFU[b]	PFU	CFU			No. of fractions	Sucrose concn range	Percentage of recovered infectivity	Ratio of infectivity CF
	rev/min	*liters*	*liters/hr*	*min*					%	%				
1	30,000	1.74	1.2–2	30	$10^{5.2}$	4	$10^{2.7}$	4	99.7	100	2	36–38	70	$10^{5.1}$
2	40,000	1.5	5	60	$10^{3.7}$	16	$10^{2.7}$	≧32	90.0	100[d]	—		—	$10^{4.8}$
3	30,000	2.4	3	120	NA[c]	NA	$10^{3.3}$	16	—	—	3	37–44	90	$10^{4.6}$
4	30,000	2.7	1.8	60	$10^{5.2}$	10	$10^{3.5}$	10	98.0	20	—		—	—
5	30,000	5.0	2.3	60	NA	NA	$10^{2.2}$	NA	—	—	3	39–44	66	$10^{5.02}$
6	25,000	3.4	3.4	20	$10^{5.8}$	10	$10^{3.9}$	10	98.8	30	7	40–52	80	$10^{5.}$
7	40,000	10.4	4.0	30	$10^{6.5}$	10	$10^{3.9}$	10	99.6	38	—		—	—
8	40,000	10.4	4.0	30	$10^{6.4}$	10	$10^{3.8}$	20	99.6	100[e]	3	40–42	92	$10^{4.9}$

[a] Plaque-forming units per milliliter.
[b] Complement-fixing units. Reciprocal of the complement-fixing antigen titer per 0.025 ml.
[c] Not available.
[d] 50,000%.
[e] 300%.

fectivity, and CF antigen distribution obtained when a larger volume (10 liters) of harvest fluid was processed. The results were similar to the results of experiments with smaller volumes except that a higher titer of virus was present in the infectivity peak. The results shown in Fig. 3 are also listed in run no. 8 in Table 1.

Table 1 summarizes experiments in which RS virus was isolated in the B-IX zonal rotor. All experiments resulted in nearly complete removal of virions from the flow-through stream. In all experiments, 66 to 92% of the banded virions were found to be centered in about 40% sucrose (density, 1.18 g/cc). The infectivity peaks ranged from 38 to 45% sucrose. Most of the CF antigen recovered in the gradient was coincident with the infectivity peak. Ratios of infectivity to CF antigen are also shown in Table 1.

Table 2 shows the clean-out efficiency (per cent removal of virions from flow-through stream) at different flow-through rates at a constant rotor speed of 40,000 rev/min. There was no significant difference in clean-out when the flow rate varied from 1.2 to 4.15 liters/hr. Clean-out efficiencies at 30,000 rev/min do not appear to be significantly different from those observed at 40,000 rev/min (see runs 1, 4, and 6 of Table 1).

Preliminary biophysical analysis of the particular material remaining in the effluent of the B-IX rotor but trapped by the B-V rotor indicated the presence of at least two species of particles in the analytical ultracentrifuge. The fastest-moving fraction had an observed S_{20w} of 17 and the slowest one had an observed S_{20w} of about 5. Material isolated from control HEp-2 cultures also contained 17*S* and 5*S* fractions. In both cases, the 17*S* material was present in smaller quantities.

The distribution of total protein within the recovered gradient shown in Fig. 3 was also determined. There is no increase in protein (nitrogen) in the fractions containg the peak of infectivity. The total purification of 95% of the virions in this infectivity fraction (Fig. 3) on the basis of protein is 1,100 times. The infectivity at the center of the zone shows a concentration of 660 times.

TABLE 2. *Cleanout of infectious RS virus in B-IX rotor at 40,000 rev/min*

Flow-through rate (liters/hr)	Titer of effluent[a] (PFU/0.2 ml)	Infectivity removed from stream (%)
4.15	2×10^3	99.7
4.20	8×10^2	99.9
3.2	8×10^2	99.9
3.2	2×10^3	99.7
2.2	9×10^1	99.98
1.2	6×10^1	99.99
1.2	6×10^1	99.99

[a] The titer of the starting sample was 6×10^5.

DISCUSSION

The effectiveness of the B-IX rotor in myxovirus isolation has been demonstrated by the high-efficiency isolation and purification of RS virus from liter volumes of harvest culture. This process combines the techniques of continuous-flow centrifugation with isopycnic separation and results in the separation of virus particles from the majority of cell debris. The effectiveness of this separation technique is demonstrated by 99% clean-out of the virus particles from solution and by the high concentration (up to 1,000 times, depending on the volume of starting sample) of infectious virus particles recovered from the density gradient. Density gradient fractions were routinely monitored by the electron microscope (results to be presented elsewhere). However, the most important aspect of this isolation technique is that it permits recovery of infectious RS virus units after the continous-flow separation. The clean-out efficiency of RS virus in the B-IX rotor indicates that this centrifugation technique would be useful for collecting and concentrating purified material for vaccine studies.

In the isolation technique reported here, sucrose was used as the density gradient material. Preliminary studies indicate that similar resolution may also be obtained with potassium citrate or cesium chloride gradients. Sucrose was used in this study, however, since materials prepared in sucrose may be used for the preparation of experimental vaccines.

The efficiency of removal of particles (clean-out) at a given rotor speed and flow-through rate is a function of the sedimentation coefficient of the particles to be isolated. Estimation of sedimentation coefficients for RS particles from the clean-out data suggests a range of 500*S* to 1,500*S*. This wide range may be attributed to the pleomorphism of the particles.

The comparatively large sedimentation coefficients of RS virus permit the particles to be recovered in the B-IX rotor at a rate and amount which may be useful in vaccine production. Assuming that 95 to 99% of appropriately grown RS virus particles were required to be removed from the harvest fluid, the flow-through rate may be as high as 7 to 10 liters/hr. On the basis of a preliminary study in which water was used instead of harvest fluid, 25 liters can be pumped through this rotor under conditions required for RS isolation. It is estimated that at least

twice this volume of harvest fluid could be processed in a single run without depleting the gradient. In practice, the liter volume limit of this isolation procedure is dependent upon the flow-through rate, the type and amount of gradient material in the rotor, the temperature of the gradient, and the sedimentation coefficient of the virus. Gradient material is continually washed out of the rotor by the effluent stream in an amount and at a rate which depends on the diffusion or mixing of the gradient into the flow stream. The comparatively low diffusion coefficient of sucrose at 5C, as compared with that of CsCl, makes this gradient material quite useful for the processing of large volumes of myxovirus harvest fluid in those instances where the myxovirus can be banded isopycnically in sucrose.

Most of the virions captured by the rotor banded at a common buoyant density in sucrose, and the peak fractions always contained 100 or more times the amount of infectivity in the other fractions. However, a few infectious particles (at least 1% of the total captured) were always detected in the remaining gradient fractions. The reasons for this are not clear. Since particle separations by this technique are based finally on buoyant density, it follows that these particles may vary in composition. This would suggest that such intact particles may contain either a higher or lower percentage of ribonucleoprotein than the particles recovered in the infectivity peak. Attachment to cell debris was not evident.

The small apparent density differences between infectivity peaks in various experiments are attributed primarily to averaging the density in each of the 20- or 25-ml gradient fractions for reference points. In the gradients used in these experiments, the variation could be $\pm$ 3%. The banding density of RS virus in sucrose-PBS gradients is considered to be 1.18 to 1.19.

Complement-fixing antigen was found to be coincident with the infectivity peak, suggesting that this antigen is primarily virus-associated. It is possible, however, to separate part of the CF activity from the infectivity by a second isopycnic banding over another gradient material, such as potassium citrate or cesium chloride. The small amount of CF antigen found near the rotor core (fractions 2 to 5) is attributed to the trapping of small virus subunits which did not have time to band isopycnically.

The purification of RS virus by this continuous-flow technique thus occurs simultaneously with virus isolation and concentration. However, some contamination of the virus zone by cellular debris does occur, as shown by the absorbancy profiles shown in Fig. 2a and 2b. Calculated purity, as the basis of protein nitrogen, was found to be 1,100 times that of the starting material. Electron microscopic evidence of the degree of purity will be presented elsewhere.

The results presented and discussed above indicate that the B-IX continuous-flow zonal rotor is a useful tool for the isolation and purification of respiratory syncytial virus. By a single rapid technique, quantitative recovery of infectious virus can be achieved. The availability of large amounts of purified RS virus make possible experimental studies on the selective disruption of infectious virus and the isolation of viral subunits.

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