Acta Medica Okayama

Volume 32, *Issue* 4 1978 *Article* 2

AUGUST 1978

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KEYWORDS: respiratory syncytial virus, purification

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*PMID: 153087 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **32**, (4), 265—272 (1978)

RESPIRATORY SYNCYTIAL VIRUS I. CONCENTRATION AND PURIFICATION OF THE INFECTIOUS VIRUS

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Received March 23, 1978

Abstract. Respiratory syncytial (RS) virus can be purified without losing its infectivity provided that each step of purification is carried out using NT buffer containing over 20% sucrose. Firstly, the virus grown on HES cells is efficiently removed from the culture fluid by precipitating with polyethylene glycol (PEG) 6,000, and the precipitate is suspended in a small amount of 20% sucrose-NT buffer, which results in about a 24-fold concentration of the original material. Then this suspension is centrifuged through 30% sucrose-NT buffer to obtain pellets, which are again suspended in 20% sucrose-NT buffer. This suspension is further centrifuged by discontinuous and linear sucrose density gradient. Finally, the specific infectivity of the purified virus was increased about 3,000-fold over that of the original material.

Key words: respiratory syncytial virus, purification

RS virus is a virus that induces very severe respiratory disease in infants (1). This virus, which in many respects resembles the paramyxoviruses (2–4), is classified in the paramyxoviridae family (5). However, it has not been fully characterised because of the size of its nucleocapsid (6–8), its inability to cause hemagglutination (9), its lack of neuraminidase activity (10) as well as the difference in the known polypeptide components (11).

Such points will become clearer with further study of its biochemical characteristics and morphogenesis. The reason for the lack of progress in this field of study is that it is very difficult to concentrate and purify intact RS virus because of its instability. Recently, Wunner and Pringle (11) and Levine (12) obtained partially purified virus and reported on the viral protein thereof. In both cases, the study was conducted with a small amount of isotopically labeled virus. For examination of the relationship between viral protein and its biological activities, large amounts of purified virus are required. So it is necessary to develop a more suitable method for the concentration and purification of large amounts of the virus.

RS virus is sensitive to changes in the temperature and pH as well as to the freezing-thawing (13). Its infectivity is also markedly reduced in water and

buffer solution (14, 15). However, fairly effective stabilizers have been found. Inorganic salts such as 1M NaCl, 1M $MgSO_4$, 1M Na_2SO_4, and 1M glucose or 1M sorbitol are good stabilizers of RS virus. It is also known that this virus can be kept stable in 44.5% sucrose solution at 4°C and −70°C for a long period of time (16, 17).

Therefore, the author studied the stabilizing conditions of sucrose suited to the medium used for the purification of RS virus and methods of concentration and purification.

MATERIALS AND METHODS

Virus. The long strain of RS virus used was donated by Dr. Reisaku Kono of the National Institute of Health, Tokyo. Stock virus was prepared in HES cell cultures.

Cell culture. HES cells, derived from human embryonic skin and established in our laboratory, were cultured in a roller bottle. The cells were grown in Eagle's minimal essential medium (MEM) supplemented with 3% fetal calf serum (FCS), and the infected cells were maintained in MEM containing 2% FCS.

Virus survival experiment. Sucrose solutions of 10%, 15%, 20%, 30%, 40% (w/w) were prepared in NT buffer solution composed of 0.15 M NaCl and 0.05 M Tris (hydroxyl methyl) aminomethane-chloride (pH 7.5). The stock virus was diluted with NT buffer and sucrose solution of each concentration in the ratio of 1 : 30, and left standing at 4°C. Thereafter the infectivity titer was determined every hour.

Virus propagation. HES cells were infected at a multiplicity of approximately 1 PFU/cell. After 2 h of adsorption at 33°C, the cells were further incubated at 33°C following the addition of maintenance medium. At about 36 h later the infected culture medium was harvested.

PEG used for concentration. Using NT buffer, 50% (w/v) polyethylene glycol 6,000 (Nakarai Chemical Products, Osaka) solution was prepared.

Titration of virus infectivity. The plaque method described by Kisch (18) was employed with some modifications. The confluent HES cells in 60 mm plastic petri dishes were inoculated with 0.2 ml of virus appropriately diluted in Eagle's MEM containing 0.5% gelatin. After 2 h at 33°C, cultures received 5 ml of agar overlay medium, which consisted of Eagle's MEM containing 5% FCS and 0.8% agar, and were then incubated at 33°C. After 4 days at 33°C, an equal amount of identical agar overlay medium was added. Finally 5 ml of overlay medium containing 0.01% neutral red were added on the 8th day after inoculation and the plaques were counted on the following day.

Protein determinations. With bovine serum albumin as the standard protein measurements were made by the method of Lowry *et al.* (19).

RESULTS

Effects of the sucrose concentration on the stabilization of RS virus. The inactivation curve was studied at 4°C in the presence of 10–40% sucrose (Fig. 1).

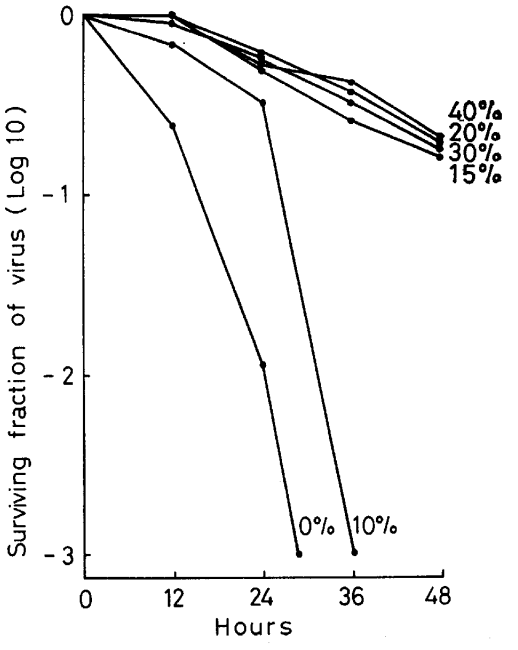

Fig. 1. Effect of concentration of sucrose on RS virus infectivity at 4°C.

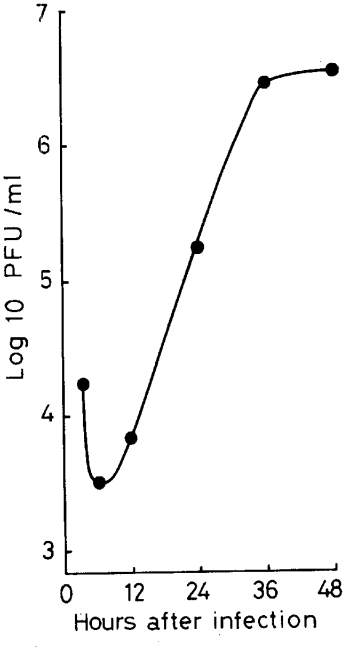

Fig. 2. Growth curve of RS virus in HES cell culture.

The half-reduction time of the infectivity titer was 7.7 h in NT buffer only, 17 h at a sucrose concentration of 10%, but it was 24 to 26 h at a sucrose concentration of 15% or over. Moreover, the inactivation rate at the 12 h incubation time was 76.1% in NT buffer alone, 30.4% in 10% sucrose-NT buffer, but above a concentration of 15% sucrose-NT buffer it was less than 7.6% in every case. Thus as long as the entire procedure of concentration and purification is conducted within 12 h at 4°C, with a sucrose concentration of over 15%, the virus remains stable.

Multiplication of RS virus in roller bottles. The release of infectious progeny started 6 to 12 h after inoculation, increased exponentially up to 36 h, and reached its peak at the 48th h (Fig. 2). By 36 h a cytopathic effect, mainly in fused cells, could be observed in the complete cell sheet. By 48 h a considerable number of cells became detached from the glass wall. Therefore, the culture medium at the 36th h of cultivation is most suitable as substrate for concentration and purification.

The concentration by PEG. The culture fluid was immediately centrifuged at 5,000×g for 10 min to remove large cell debris (Fig. 3). The following procedures were all conducted at 4°C or on ice. To the supernatant 50% PEG 6,000

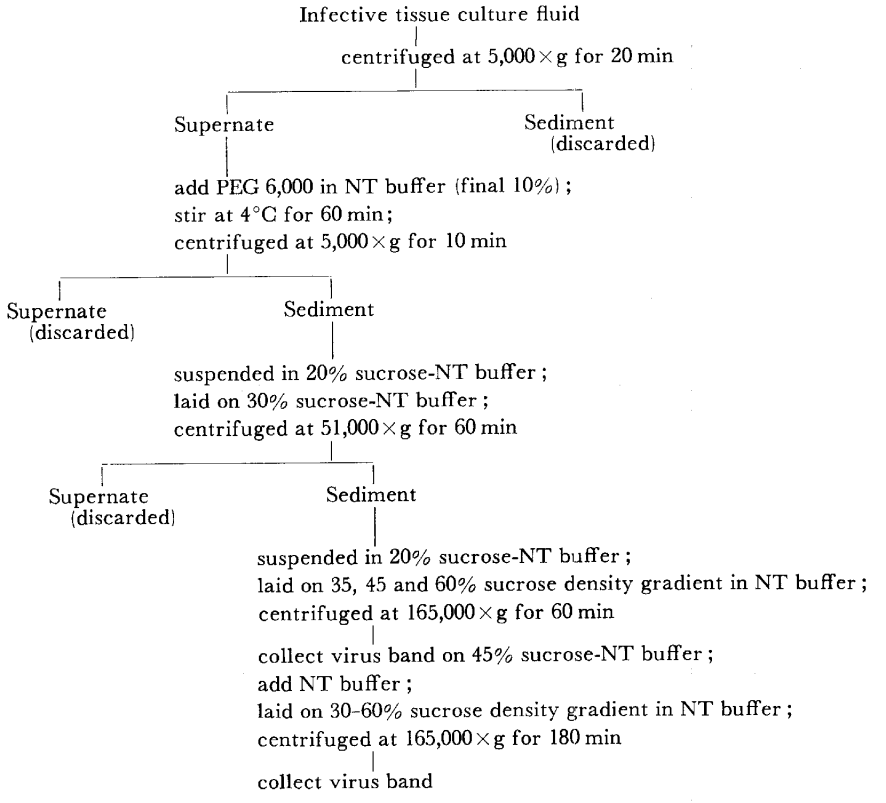

Fig. 3. Schematic representation of the procedure used for the purification of RS virus growth in HES cell cultures.

TABLE 1. PURIFICATION OF RS VIRUS

Sumple	Vol. (ml)	PFU/ml (10^6)	Amt of Protein (mg/ml)	PFU/mg of Protein (10^6)	Recovery (%)
Infective tissue culture fluid	1,200	5.5	2.40	2.2	100
Cocentration with PEG	50	121	3.74	32.4	92
Resuspended pellet after high-speed centrifugation	1	6,300	1.29	4,900	95
Band after centrifugation in discontinuous sucrose density gradient	0.5	4,400	0.77	5,700	33
Band after centrifugation in linear sucrose density gradient	0.75	1,700	0.25	6,800	19

was added to make the final concentration 10%, and stirred for 60 min. Then it was centrifuged at 5,000×g for 10 min, and the sediment was suspended in 20% sucrose-NT buffer with a dounce-homogenizer in 1/24 of the original material. Infectious virus collected amounted to 92% of the original material (Table 1).

Purification by ultracentrifugation. The material concentrated with PEG 6,000 was centrifuged through 30% sucrose-NT buffer (Fig. 3). There was hardly any loss of infectious virus at this step of the purification (Table 1). Virus suspension was layered onto 35, 45 and 60% sucrose-NT buffer gradient, then centrifuged at 165,000×g for 60 min. A distinct band was observed on the boundary surface between 35% and 45% sucrose-NT buffer. This virus band was collected, layered on to a linear 30–60% sucrose- NT buffer gradient, then centrifuged at 165,000×g for 180 min. One distinct band when formed slightly below the middle was collected from the bottom of the tube by fractionation. The infective virus was located in the region of density 1.18–1.20 g/cm^3, and its peak was at 1.19 g/cm^3 (Fig. 4). Purified virus was obtained by pooling fractions Nos. 12, 13 and 14. The infective titer of purified virus was 1.7×10^9 PFU and

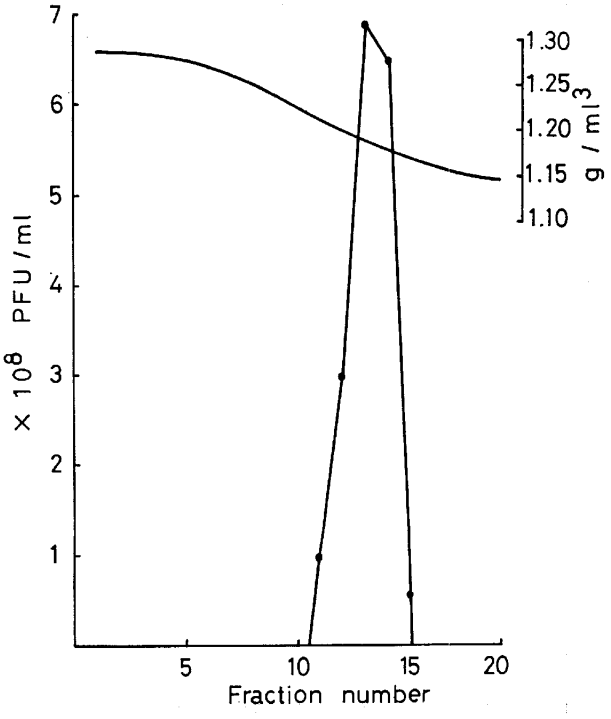

Fig. 4. Sucrose gradient analysis of RS virus.

its recovery rate was 19%, while its protein concentration was 0.25 mg/ml. The ratio of infectivity to protein content was $6,800 \times 10^6$ PFU/mg in the purified virus in contrast to 2.2×10^6 PFU/mg in the original material, showing an increase of about 3,000-fold.

DISCUSSION

In the concentration method of RS virus by Kanarek and Tribe favorable results were obtained by using PEG 6,000 as with other paramyxoviruses (20). This method not only enables one to concentrate virus within a short period of time, but also eliminate a large amount of the protein in the culture medium, so it is a very useful first step in purification. In the present experiment after the completion of this step the specific infectivity rose 15-fold.

No attempt was made to purify infectious RS virus until the experiments of Wunner and Pringle (11). They used the method of resuspending the pellet concentrated with PEG 6,000 in NTE buffer or HEPES buffer, and immediately centrifuging by isopycnic banding in sucrose or metrizamide. However they described only a partial purification, and while the recovery rate at each step remains unclear, resuspension in NTE buffer and HEPES buffer as well as a long time-centrifugation must have resulted in considerable viral inactivation. Levine's purification (12) of RS virus using Hank's balanced salt solution (HBSS) demonstrated that the infectivity of RS virus did not diminish by treating it at 4°C for 18 h. However, Hambling (13) found that the infectivity of RS virus fell to 1/10 in HBSS after 48 h, and we also observed a considerable reduction in infectivity after 12 h in HBSS, hence we consider HBSS to be a poor stabilizer. The purification procedure employed by Levine requires about 60–69 h and during that time a considerable inactivation must have occurred.

For purification without loss of infectivity, it is important that a medium suitable for purification be selected from among the stabilizers of RS virus.

Inorganic salts such as $MgSO_4$ and NaCl at high concentration (14, 15), are known to be stabilizers of RS virus, but are not so suitable since they induce agglutination of intracellular structural conponents and inhibit fractionation. On the other hand, according to Law and Hull (16), a virus stock preserved for a long period of time at −70°C in 44.5% sucrose solution was very stable and even at 4°C it was stable for up to 24 days. Tai *et al.* (17) also state that the infective titer is best at a preservation temperature of −60°C in 45% sucrose, and that the infective titer at 4°C does not decrease for 7 days. Sucrose, which seems to be a suitable stabilizer of RS virus as mentioned above, has been widely employed for the purification and fractionation of viruses and cellular components during ultracentrifugation. With this in mind the author studied the use of sucrose during the purification of RS virus. Since the minimal time required for the

purification of virus is 12 h, the minimal sucrose concentration which would not diminish the infectivity was studied and it proved to be over 15%. Therefore, 20% sucrose-NT buffer was used as the suspension solution and in all other steps over 20% sucrose-NT buffer was used.

In the step where the virus concentrated with PEG 6,000 was centrifuged through 30% sucrose-NT buffer, there was no decrease in the infectivity, and it was possible to concentrate to 50-fold. Moreover, when a considerable amount of cellular components remained on 30% sucrose-NT buffer the specific infectivity rose 151-fold, indicating that it is an excellent step for concentration and purification. To remove as much cellular components as possible the purification was accomplished by discontinuous sucrose density gradient, before the final step of purification with a linear sucrose density gradient. At this step there was a faint band on 35% and 60% sucrose-NT buffer, and it was possible to remove some cellular components from the virus band at 45% sucrose-NT buffer stage. The protein content was reduced about 60% in the earlier steps. The recovery rate of infectious virus is high and the infectivity titer increased in the final step with a linear sucrose density gradient. Accordingly, the method described here proved to be effective for the concentration-purification of infectious RS virus.

For the elucidation of the relationship of virus structural component to their biological activity in the future large amounts of purified virus will be required. The present purification procedure suits this requirement.

Acknowledgment. The author is indebted Prof. J. Tawara, Dr. H. Kumon and Dr. K. Akatsuka for providing many helpful suggestions.

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