A New, Rapid Procedure for the
Concentration of C-type Viruses from Large Quantities of Culture Media: Ultrafiltration by Diaflo Membrane and Purification by Ficoll Gradient Centrifugation

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SUMMARY

The Amicon TCIR recycle system with Diaflo PM30 filters provided a rapid, simple and inexpensive method for the concentration of baboon endogenous virus from a large quantity of culture medium from chronically infected cells. The recovery of infectivity and virion RNA-dependent DNA polymerase activity was substantially better than after the conventional concentration methods of ultracentrifugation or precipitation with ammonium sulphate or polyethylene glycol 6000. The combination of ultrafiltration and Ficoll gradient centrifugation is recommended as a general procedure for the purification of fragile C-type viruses.

INTRODUCTION

For many studies infectious C-type viruses must be concentrated from large quantities of culture media. Isopycnic banding in an ultracentrifuge equipped with a continuous flow system has often been employed for the purification of C-type viruses (Barringer, 1966). However, this procedure requires expensive equipment and tedious assembly. Relatively stable C-type viruses (e.g. some avian C-type viruses) are often concentrated by precipitation with ammonium sulphate or polyethylene glycol (Duesberg et al. 1968; Green et al. 1970), but these procedures tend to inactivate more fragile C-type viruses.

In this report we describe the concentration of C-type viruses by a simple and relatively inexpensive filtration method with Diaflo membrane, which did not cause a significant loss of infectivity or activity of virion-associated RNA-dependent DNA polymerase (RDPase) (Baltimore, 1970; Temin & Mizutani, 1970). Using the baboon endogenous virus, we have compared Diaflo ultrafiltration as a concentration method with concentration by ultracentrifugation and by precipitation with ammonium sulphate or polyethylene glycol. Later in this paper we describe the purification procedures of the virus from the concentrated medium by sucrose and Ficoll gradient centrifugation. An isopycnic banding in the Ficoll gradient gave a higher yield of highly purified viruses than did banding in sucrose.

METHODS

Virus and cell cultures. A human rhabdomyosarcoma cell line, A204 (Giard et al. 1973), was chronically infected with baboon endogenous virus M7 (BaEV) (Benveniste et al. 1974). The infected cells were cultured with the medium, RPMI1640 (GIBCO) supplemented with
10% foetal calf serum (GIBCO). At the middle or late logarithmic growth phase of the infected cells in glass roller bottles (597 cm²) or in bulk culture vessels (8500 cm², Sterilin) [House, 1973], the media were collected twice a day from the cultures and stored at −40 °C.

**Ultrafiltration.** Amicon's TCIR recycle system and Diaflo PM30 membrane filters (diam. 150 mm; average pore size 0.22 μm) were used in the cold (4 °C). The other experimental conditions are described under Results.

**Ammonium sulphate and polyethylene glycol precipitations.** Samples of the thawed medium (70 ml) were centrifuged at 2500 g for 20 min at 2 °C, and 70 ml of either pre-cooled saturated ammonium sulphate or 20% (w/v) polyethylene glycol (PEG) 6000 (Wako Pure Chemicals, Osaka, Japan) in 1 M-NaCl (pH 7) were added to the supernatants. After incubation on ice for 90 min, the mixtures were centrifuged at 2300 g for 30 min at 2 °C. The pooled precipitates were dissolved in 3.5 ml of 10 mM-tris-HCl buffer, pH 7.2, 0.1 M-NaCl, 1 mM-EDTA (TNE buffer).

**Virion-associated RNA-dependent DNA polymerase (RDPase) assay.** Thirty microlitres of diluted or undiluted samples were treated with 0.01% Brij-58 (Nakarai-Chemicals, Osaka, Japan) and used for an RDPase assay with poly rA: oligo dT (12-18) (PL Biochemicals, Wisconsin, U.S.A.) as the template-primer and 3H-TTP (NEN Co., Massachusetts, U.S.A.) as the substrate. The procedures of the RDPase assay were a modification of those described by Spiegelman et al. (1970) and further details will be published elsewhere (T. Tamura & T. Takano, unpublished data). Briefly, the reaction (60 min at 37 °C) was stopped by adding 67 mM-EDTA, the reaction mixture was applied to DEAE-cellulose filters (Whatman, DE-81); after drying, the filters were washed to remove unincorporated 3H-TTP and the incorporated radioactivity was determined. The reactions were carried out under conditions such that the amount of 3H-TMP incorporated was dependent on the amount of virus added and the reaction proceeded linearly up to 90 min. One enzyme unit was defined as the amount of activity that catalyses the incorporation of 1 pmol of TMP per 60 min at 37 °C.

**Infectivity assay.** A204 (1 × 10⁵) cells were inoculated in each well of culture plates (Linbro, 2 cm²/well) and infected with 0.2 ml of samples at various dilutions. On the 5th day after infection, 30 μl of the medium from each well were used for the RDPase assay. The RDPase activities were plotted on logarithmic section paper against the dilution factors which were read at the 0.3 enzyme units curve. The reciprocal of the value was defined as the tissue culture infecting dose of each sample.

**Protein.** Protein was determined from the absorbance at 260 and 280 nm, as described by Layne (1957).

**Isopycnic banding of BaEV in sucrose and Ficoll gradient centrifugation.** The medium concentrated by ultrafiltration was centrifuged at 68000 g for 60 min at 2 °C. The precipitates were resuspended with 1/54 vol. of TNE buffer. This sample was applied for the purification of the virus by centrifugation in sucrose and Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden; Nowinski et al. 1967) gradient solutions. The Ficoll solution was used after dialysis against distilled water. Sixteen ml of linear gradient solutions of sucrose (15 to 50% w/v) or Ficoll (8 to 35% w/v) were prepared in TNE buffer. One ml of the concentrated medium was applied on these gradient solutions and centrifuged at 65000 g for 4 h at 2 °C in a Beckman SW-27 (16 ml tubes) rotor. After centrifugation, 0.8 ml samples of the gradient solution were fractionated from the bottom of each centrifuge tube. Refractive index, absorbance at 280 nm and the RDPase activity of each fraction were determined.
Concentration and purification of C-type virus

Table 1. Concentration of baboon endogenous virus*

<table>
<thead>
<tr>
<th>Vol. (ml)</th>
<th>Infectivity</th>
<th>RDPase activity</th>
<th>Dilution factor at 0.3 units†</th>
<th>Tissue culture infecting dose [1/A]</th>
<th>Total infecting dose [1/A] × vol. (ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td></td>
<td>Ultrafiltration by Diaflo membrane PM30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7440</td>
<td>6.0 × 10⁴</td>
<td>(100)‡</td>
<td>5.9 × 10⁻³</td>
<td>169</td>
<td>1.3 × 10⁸</td>
<td>(100)‡</td>
</tr>
<tr>
<td>After</td>
<td>750</td>
<td>7.4 × 10⁴</td>
<td>123</td>
<td>5.0 × 10⁻⁴</td>
<td>2.0 × 10⁻¹</td>
<td>75</td>
</tr>
<tr>
<td>Filtrate</td>
<td>6490</td>
<td>1.7 × 10⁴</td>
<td>28.3</td>
<td>2.0 × 10⁻¹</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Precipitation with ammonium sulphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>70</td>
<td>5.7 × 10²</td>
<td>(100)‡</td>
<td>5.9 × 10⁻³</td>
<td>169</td>
<td>1.2 × 10⁴</td>
</tr>
<tr>
<td>After</td>
<td>3.5</td>
<td>6.0 × 10²</td>
<td>105</td>
<td>1.3 × 10⁻²</td>
<td>77</td>
<td>2.7 × 10²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Precipitation with polyethylene glycol 6000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>70</td>
<td>5.7 × 10²</td>
<td>(100)‡</td>
<td>5.9 × 10⁻³</td>
<td>169</td>
<td>1.2 × 10⁴</td>
</tr>
<tr>
<td>After</td>
<td>3.5</td>
<td>3.9 × 10²</td>
<td>68</td>
<td>1.7 × 10⁻²</td>
<td>59</td>
<td>2.1 × 10²</td>
</tr>
</tbody>
</table>

* Aliquots of the same sample of the cultured medium were used for these three different concentration procedures.
† These are read on Fig. 1 as described under Methods.
‡ The numbers in the parentheses indicate 100% of the recovery defined from the total RDPase activity or infectivity of the samples initially applied to each concentration procedure.

RESULTS

Concentration of baboon endogenous virus by ultrafiltration

The culture medium of A204 cells chronically infected with BaEV was applied to the TCIR ultrafiltration system with Diaflo membrane PM30 under nitrogen gas pressure of 1.0 kg/cm². After 15 h of filtration, 6490 ml out of the initial vol. of 7440 ml were filtered through the system, and the vol. of the fluid remaining in the reservoir was 750 ml, which corresponds to 10.1% of the initial vol. The average flow rate was about 433 ml/60 min and the flow rate decreased gradually during filtration. The pattern of the filtration rate did not change with differences in the vol. initially applied, nor with differences in N₂ gas pressure (1.5 to 2.5 kg/cm²).

The recovery of RDPase activity in the concentrated fluid and filtrate was 123 and 28%, respectively (Table 1). The recovery of the infectivity of the concentrated media was found to be 115%, as judged by the tissue culture infecting doses described under Methods (Fig. 1 and Table 1). The excess recovery of both infectivity and RDPase activity may be due to the unmasking effect of the dissociation of virions which had aggregated with each other or with cellular fragments. This interpretation is supported by morphological observations as the concentrated virions appeared intact in the electron microscope (data not shown). Clearly, the BaEV in the culture medium was concentrated by ultrafiltration without significant loss of infectivity. These results were reproducible.

Concentration of baboon endogenous virus by precipitation with (NH₄)₂SO₄ or polyethylene glycol

Concentration of BaEV by precipitation with ammonium sulphate, polyethylene glycol (PEG) 6000, or by ultracentrifugation was carried out as described in the Methods. The recoveries of the RDPase activities by these procedures are shown in Table 1, and the
Fig. 1. Infectivity of concentrated baboon endogenous virus. The samples of the experiment shown in Table I were assayed by the procedures described under Methods. ●, sample before the concentration; □—□, sample concentrated by ultrafiltration; ▽—▽, by ammonium sulphate precipitation; ▲—▲, by PEG 6000; and □—□, the filtrate of the ultrafiltration.

infectivities of BaEV in these preparations are shown in Fig. 1. It is clear that the recovery of the infectivity in these preparations was much less than with BaEV concentrated by ultrafiltration, although the recovery of the RDPase activity was relatively high. The inactivation of the BaEV was marked when concentrated with ammonium sulphate or PEG 6000.

Purification of the concentrated BaEV by isopycnic banding

The medium concentrated by ultrafiltration was pelleted and the precipitates were resuspended in 1/54 vol. of the TNE buffer. This concentrated virus preparation, which still contained a lot of serum proteins from the medium and cellular debris, was then applied to sucrose or Ficoll gradient solutions. After centrifugation, the refractive index, the absorbance at 280 nm and the RDPase activity of each fraction were determined (Fig. 2). The peaks of the RDPase activity which represented the band of the purified virions were located at the density of 1.14 and 1.075 g/ml in the sucrose and Ficoll gradient solutions (TNE buffer), respectively. The density of BaEV in the Ficoll gradient solution was similar to that in a Percoll (Klingeborn & Pertoft, 1972) gradient solution (data not shown). Therefore, the high density of the virus in the sucrose solution might be due to a higher osmotic pressure of such small molecules as sucrose compared with that of Ficoll, a solution of polymers of sucrose. BaEV might preserve a density closer to that of the physiological state in the Ficoll solution.

The fractions shown in the parentheses of Fig. 2 were pooled and the virions of these pooled fractions were concentrated by pelleting at 68 000 g for 60 min at 2 °C, in a Beckman SW27 rotor (16 ml tubes). The precipitates of each peak fraction were resuspended with 0.5 ml of TNE buffer. The purity of the virus preparation in each peak of these gradient centrifugations was determined from the relative ratio of the RDPase activity to the amount of protein of these pooled, concentrated fractions. Also, the recovery of the RDPase activity
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Fig. 2. Profiles of the isopycnic bandings of baboon endogenous virus in Ficoll and sucrose gradient solutions. Samples of the culture medium concentrated by ultrafiltration were applied on each gradient solution. (a) Ficoll density gradient centrifugation; (b) sucrose density gradient centrifugation. ○--○, the RDPase activity; •---•, absorbance at 280 nm; and ---, the density of the solution.

after isopycnic banding was determined. The recoveries of the RDPase activity in these pooled samples were 70 and 18% of that in the initially applied sample, in the sucrose and Ficoll gradient centrifugations, respectively. The purity of the peak of the sucrose and Ficoll gradient centrifugations were 508 and 490 units per mg protein, respectively. From these results, the Ficoll gradient centrifugation clearly gave a highly purified preparation of BaEV with a better recovery than did the sucrose gradient centrifugation.

DISCUSSION

By using the baboon endogenous virus as a representative of fragile C-type viruses, it is clearly shown that the combination of Diaflo ultrafiltration concentration and purification by Ficoll gradient centrifugation gives a highly purified, concentrated preparation of C-type viruses from large quantities of culture media.

Rhim et al. (1969) concentrated murine leukaemia and sarcoma viruses by a non-recycle version of the Diaflo ultrafiltration system, model 50, with UM10 membrane (average pore size, 0.14 μm) and reported a relatively higher recovery of the infectivity of the viruses. However, when we used another non-recycle type Diaflo ultrafiltration system, TCF10, with membrane XM100A (diam. 90 mm; average pore size 0.53 μm) with a reservoir (5 l), after four- to fivefold concentration the BaEV recovered was only 30 to 40% of the initial total RDPase activity. In addition, the flow rate was much slower (80 to 90 ml/h). The concentrated fluid seemed to form a thin film on the filtration membrane during the operation and a large amount of protein deposit was observed on the surface of the membrane. If recycling accessories are available the Diaflo ultrafiltration system, TCF10 seems to be adequate for concentrating up to 5 l at one time. The TCIR ultrafiltration system can process as much as 19 l. The system can be autoclaved without difficulties, and the filter membrane can be kept
in 50% ethanol and reused after occasional washings with neutral detergent. The TCIR ultrafiltration system is completely air-tight and closed, so one can operate safely without any contamination by the aerosols of biohazardous viruses.

Bronson et al. (1976) reported that Rous sarcoma virus was concentrated and purified intact by centrifugation or by PEG precipitation followed by pronase treatment. These procedures, however, seem to inactivate the infectivity of a fragile C-type virus like BaEV (Table I). While they described that ultrafiltration by the membrane XM300 gave a poor recovery of the RDPase and the focus-forming activity of the Rous sarcoma virus, they did not mention the recovery of the RDPase activity in the filtrate fraction. When we used the membrane XM300 for the concentration of BaEV, most of the RDPase activity was recovered in the filtrate, but not in the concentrated medium (data not shown). Thus, we suspect that the membrane XM300 might not be suitable for the ultrafiltration concentration of C-type viruses.

In conclusion, the following procedures for the purification of C-type viruses are recommended; the culture media of the virus-producing cells are concentrated by the Amicon TCIR ultrafiltration system with the Diaflo membrane, PM30, and recycling accessories. These concentrated preparations are isopycnically banded in 8 to 38% Ficoll gradient. From our results, the purification by the Ficoll gradient centrifugation was much better than that by the sucrose. However, the recovery of the virus by the Ficoll gradient centrifugation is still low (18% of the initially applied RDPase activity). We do not have any evidence to explain this low recovery. Other agents for making density gradients, for example, Percoll (Klingeborn & Pertof, 1972), might be useful to yield a higher recovery of highly purified virus preparations.

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REFERENCES


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