Lymphocytic Choriomeningitis Virus

I. Concentration and Purification of the Infectious Virus

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Two procedures for the purification of infectious lymphocytic choriomeningitis virus from cell culture fluid have been developed. If large quantities of very pure virus are to be prepared, infected L cells are maintained with a medium supplemented with calf serum, the proteins of which have been largely removed by pretreatment with polyethylene glycol. Two days after infection of the cultures, the media are collected and the virus is concentrated by treatment with polyethylene glycol 40,000. Purification with a 10,000-fold increase of specific infectivity is achieved with steric chromatography on controlled-pore glass beads with pore sizes of 42 to 44 nm and centrifugation in density gradients prepared with amido trizate. An alternative method begins with precipitation of the virus from infected cell culture medium with zinc acetate, followed by controlled-pore glass chromatography and density centrifugation in a discontinuous sucrose gradient. Purification thus obtained is 200-fold in terms of specific infectivity.

Infection of Mus musculus with lymphocytic choriomeningitis (LCM) virus has long been regarded as an exceptionally informative model for studying numerous aspects of the interaction between a virus and its animal host (14). Yet the phenomena of interest, which are associated with acute or persistent LCM virus infection of the mouse, are only partly understood. One reason for the slow progress may be seen in the limited knowledge we have of the structure of the virion. Because of the extraordinary lability of the infectious property of LCM virus, purification as a prerequisite for studying its biochemical and antigenic composition has been a difficult task. In this communication we report a procedure allowing the preparation of large amounts of highly purified LCM virus which has retained most of its infectivity.

MATERIALS AND METHODS

Virus. LCM virus strain WE (27) was used throughout. Its quantification was based on infectivity and was performed either by means of a quantal micromethod (9) or a plaque test on L cells, using methyl cellulose as the solidifying substance in the overlay medium (M. Popescu and F. Lehmann-Grube, submitted for publication). Titters are expressed as mean infective doses and PFU, respectively.

L cells, clone 929, were freed of mycoplasma organisms by treatment with a hypotonic solution of antibiotics according to Gori and Lee (7). They were grown as monolayers in rolled glass bottles (12), each having a surface area of 1,600 cm². Growth medium consisted of minimum essential medium (6) supplemented with non-essential amino acids (15), 0.25% lactalbumin hydrolysate, and 3% heated calf serum. Infected cells were maintained in a similar medium which contained, in addition to the constituents listed, N-2-hydroxyethyl-piperazine-N'-2'-ethanesulfonic acid (29) at a final concentration of 0.01 M. If the virus was to be concentrated by polyethylene glycol (PEG), calf serum, from which the bulk of large proteins had been removed by treatment with 6% PEG 40,000, was used. The 10,000 × g supernatant still containing the PEG and approximately one-quarter of the original proteins was sterilized by filtration and stored at -20 C until use. Calf serum thus treated maintained the cells and permitted virus multiplication just as well as untreated serum. All media contained penicillin and streptomycin, 100 U and 100 µg/ml, respectively.

Because of the great lability of LCM virus (13), care was exercised to prevent loss of infectivity due to thermal inactivation. Thus, if virus had to be stored, it was snap-frozen in small samples in ampoules and kept at -70 C; for the purpose of titration virus dilutions always contained 5% heat-inactivated calf serum. All manipulations involving virus were performed at ≤5 C.

Labeling of L cells with radioactive isotopes. [3H]uridine (40,000 to 60,000 mCi/mmol) and [14C]-labeled protein hydrolysate (>45 mCi/matom of carbon) were purchased from Amersham Buchler, Braunschweig, Germany. To mark cellular RNA, uninfected incomplete monolayers were incubated with maintenance medium to which was added 5 µCi of [3H]uridine per ml. Cellular proteins were marked with 1 µCi of [14C]-labeled amino acids per ml, which was added to maintenance medium.
containing one-tenth the prescribed concentration of unlabeled amino acids. After incubation for 3 days, the media from radioactive cultures were treated with NaCl and PEG 40,000, both at final concentrations of 6%. The cells were collected, disrupted by ultrasonication, and centrifuged at 3,000 × g for 10 min. The supernatants and the resuspended precipitates resulting from PEG treatment of medium were mixed and used in combination to monitor the purification procedure to be described (see below). Samples were mixed with a Triton-based toluene cocktail, and radioactivity was measured with a liquid scintillation counter (5).

**Chromatography.** Details have been described (8). In brief, Chromodule columns (H. Hözel, Dorfen, Germany) with dimensions of 0.8 by 100.0 cm and 1.1 by 100.0 cm (types 08 and 11, respectively) were filled with controlled-pore glass (CPG) of 43.7 actual pore size. (CPG was a gift from W. Haller, National Bureau of Standards, Washington, D.C.; now available from Electro-Nucleonics, Inc., Fairfield, N.J.) Adsorption of virus to the glass was prevented by pretreatment of the column with 1% PEG 20,000 dissolved in water (11). Buffer (GNTE) contained 0.2 M glycine, 0.2 M NaCl, 0.02 M Tris, and 0.002 M EDTA. The pH was adjusted to 7.8 with HCl. Cooling of the column itself is not required; the virus remains in the exclusion volume and passes through the column within minutes.

**Density gradients.** Sucrose was dissolved 60% (wt/wt) in GNTE buffer. Other concentrations were obtained by diluting (wt/wt) this stock solution with GNTE.

Urografin, which is a 76% solution of sodium and methyl-glucamin salts (66 and 10 parts, respectively) of N,N'-diacetyl-3,5-diamino-2,4,6-trijodobenezic acid (amido trizoate), was a gift from Schering AG, Berlin. It was further diluted with GNTE buffer. Determinations of densities were based on refractive indices, which were measured with a Zeiss-Abbé refractometer (C. Zeiss, Oberkochen, Germany) and calculated by comparison with a calibration curve, taking the temperature into account. Details of preparation and use of this newly developed gradient will be published separately (manuscript in preparation).

**Protein determinations.** Concentrations of proteins were determined according to Lowry et al. (16), using bovine serum albumin as a standard. Alternatively, the procedure of Nakao et al. (18) was employed.

**RESULTS**

In preliminary experiments, optimal conditions for growth of cells and multiplication of virus in rolling bottles were determined. Best results are obtained with light monolayers which are infected with 0.001 to 0.01 mean infective doses/cell contained in 250 ml of maintenance medium, with incubation at 37 C for 40 to 48 h and with a rolling speed of 20 rotations/h. Addition of EDTA to the medium at a final concentration of 0.005 M 2 h before harvest detaches the cells from the glass and increases virus yields two- to fivefold in both medium and cells. This phenomenon is unexplained.

To keep the initial contamination of the virus preparation with cellular constituents to a minimum, only culture media are used as starting material. Two procedures have been developed for the concentration and purification of the virus based on phase exclusion with PEG and precipitation with zinc acetate, respectively, although use of PEG has proved to be preferable.

**PEG procedure.** The procedure finally adopted is outlined in Fig. 1. Calf serum used for maintaining virus-infected cells is freed from

Collect medium from infected L-cell cultures and chill to 0 C; centrifuge 15 min at 6,000 × g.

Discard sediment. Add 6% NaCl and 4% PEG 40,000; dissolve by vigorous stirring; leave 3 to 16 h at 0 C.

Centrifuge 20 min at 6,000 × g.

Discard supernatant. Suspend sediment in GNTE-buffer to give 200-fold concentration.

Centrifuge 5 min at 2,000 × g.

Keep supernatant at 0 C; sonicate sediment; mix sediment with supernatant.

Centrifuge 15 min at 10,000 × g.

Discard sediment. Chromatograph on CPG column.

Discard rest of eluate. Collect eluate corresponding to exclusion volume.

Adjust with amido trizoate to density 1.08 g/cm³; put on discontinuous gradient, 1.12 and 1.25 g/cm³; centrifuge 60 min at 96,000 × g.

Discard rest of gradient. Collect band on 1.25-g/cm³ cushion.

Adjust to linear amido trizoate gradient, 1.08 to 1.20 g/cm³; centrifuge either 9 h at 300,000 × g or 16 h at 200,000 × g.

Collect upper band located at position corresponding to 1.137 to 1.142 g/cm³.

Chromatograph on CPG column.

Discard rest of eluate. Collect eluate corresponding to exclusion volume.

Fig. 1. Schematic representation of the PEG procedure for the purification of LCM virus from medium of infected cell cultures.
a considerable portion of its proteins by pre-treatment with PEG (see Materials and Methods). Media from infected cultures are centrifuged in the cold to remove cells and cellular debris. NaCl and PEG 40,000 are then added to give final concentrations of 6 and 4%, respectively. A precipitate is allowed to form which is sedimented by centrifugation. It is taken up in GNTE buffer, and the nonsoluble particles are broken up by sonicating. Coarse impurities are removed by centrifugation at 10,000 x g. Up to 100% of infectious virus is recovered from the precipitate when PEG 40,000 is employed at concentrations varying from 3.5 to 5%. Recovery is reduced if more or less PEG is added to the medium.

The next step consists of chromatography of the 10,000 x g supernatant using CPG with pore sizes of 42 to 44 nm. If the sample volume corresponds to at least 50% of the pore volume of the column, infectious virus can be collected in the exclusion volume without dilution. PEG and contaminants smaller than the infectious virus penetrate into the pores of the glass beads and are retarded when passing through the column.

Further purification is achieved by means of centrifugation in a discontinuous density gradient formed with amido trizoate. The eluate from the column is adjusted to 1.08 g/cm³ and put on a gradient consisting of two layers of amido trizoate in GNTE with densities of 1.12 and 1.25 g/cm³, respectively. Upon centrifugation at 5 C for 60 min at 96,000 x g in the SW27 rotor of the Beckman centrifuge, bands become visible in each interphase and light-scattering material can also be seen throughout the two upper layers. Infectious virus is concentrated five- to 10-fold in a band on top of the 1.25-g/cm³ basic layer.

The fraction containing the infectious virus has a density of about 1.17 g/cm³. It is divided into equal parts, one of which is adjusted to a density of 1.20 g/cm³ with amido trizoate and the other to a density of 1.08 g/cm³ with GNTE. From these preparations a linear gradient is mixed, resulting in an even distribution of the virus in the tube. Depending on the amount to be treated, centrifugation is either at 5 C for 9 to 12 h at 300,000 x g in 5.5-ml tubes of a Beckman SW65 rotor or at 5 C for 16 to 20 h at 200,000 x g in 12.0-ml tubes of the SW41 rotor. Two sharp bands become visible in the new exponential gradient, one at a density of 1.14 g/cm³ (upper third of tube) and one at a density of 1.20 to 1.22 g/cm³ (lower third of tube). Approximately one-half the infectious virus originally contained in the gradient is concentrated in the former. The heavy band contains only background infectivity. It is probably made up of virus-specific material, but characterization has not yet been achieved.

Centrifugation in a linear gradient followed by chromatography ends the purification procedure. The strongly UV-light-absorbing gradient material is completely eliminated from the virus-containing band by passing it through a column with CPG of 42- to 44-nm pore size. Data from the three last purification experiments performed in this series are presented in Table 1, and average results from all successful experiments are given in Table 2. The degree of purification as based on virus titrations and protein determinations is about 10,000-fold.

To have an estimate of the quantity of cellular impurities which might have slipped through the various purification steps, the following experiment was performed. Disrupted cells plus PEG-precipitated constituents of media from normal L-cell cultures labeled either with [3H]uridine or with 14C-labeled amino acids (see Materials and Methods) were added to media from unlabeled L-cell cultures harvested 46 h after infection. Radioactivity was determined at each purification step. This experiment was repeated. The averages of counts are included in Table 2. In Fig. 2 the last purification step has been illustrated in detail. It can be seen that the purified virus contains less than 0.1% of the initial uridine label and less than 1% of the initial amino acids label. There can be no doubt that this degree of contamination with cellular constituents is greatly in excess of what might be expected in the purified virus. Media from infected cultures are harvested several hours before cytopathic effects become apparent, and hence the addition of disrupted cells before purification does

Table 1. Results of the three last purification experiments of this series employing the PEG procedure

<table>
<thead>
<tr>
<th>No. of expt</th>
<th>Starting material</th>
<th>Purified material</th>
<th>PFU/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vol (ml)</td>
<td>PFU/ml</td>
<td>Vol (ml)</td>
</tr>
<tr>
<td>199</td>
<td>7,500</td>
<td>5.6 x 10⁷</td>
<td>13.5</td>
</tr>
<tr>
<td>200</td>
<td>5,000</td>
<td>10⁴</td>
<td>6.5</td>
</tr>
<tr>
<td>201</td>
<td>10,000</td>
<td>4.9 x 10⁷</td>
<td>2.0</td>
</tr>
</tbody>
</table>
drops from 40,000 L-cell cultures, centrifuged was infectious and with culture cells plus material from and text in virus what know not at all resemble the events occurring in an infected culture. On the other hand, we do not know what types of proteins are shed from cells together with infectious LCM virus.

**Zinc acetate procedure.** An alternative method is useful, especially when small amounts of material are to be processed; it commences with precipitation of virus and proteins with zinc acetate. Optimal conditions for LCM virus are essentially the same as have been described for rabies virus (28). Zinc acetate, dissolved 1.0 M in water, is added to medium from infected cultures (calf serum untreated) to give a final concentration of 0.02 M. The mixture is stirred for 20 min and then centrifuged for 20 min at 2,000 x g. The virus, together with approximately one-fourth of the proteins of the starting material, is contained in the voluminous sediment. This is dissolved in a small amount of diluent consisting of 0.5 M EDTA and 1.0 M Tris in water. Purification is accomplished with column chromatography using CPG of pore size 42 to 44 nm; infectious virus remains in the exclusion volume. Further concentration and purification is achieved by centrifugation for 2 h at 96,000 x g in a discontinuous (50, 25, and 10%) sucrose gradient. Whereas cellular impurities remain in the 25 and 10% layers, up to 40% of infectious virus is recovered from a band which forms on the 50% cushion. This band is collected and, as the last step of this procedure, sucrose is removed by CPG chromatography. Results from numerous experiments are summarized in Table 3. Final concentration of infectious virus is 50- to 100-fold, and specific infectivity is increased about 200-fold.

**DISCUSSION**

During 40 years of LCM research, satisfactory purification of the infectious virus has not been achieved, although a variety of procedures have been attempted (14, 23). One reason is the extraordinary lability of the infectious property

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**Table 2. Average results of experiments employing the PEG procedure for concentration and purification of LCM virus**

<table>
<thead>
<tr>
<th>Prepn step</th>
<th>Vol (ml)</th>
<th>PFU/ml (log10)</th>
<th>PFU/mg of protein (log10)</th>
<th>Recovery (%)</th>
<th>3H counts/min (RNA) from L cells (log10)</th>
<th>14C counts/min (protein) from L cells (log10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection of medium</td>
<td>10,000</td>
<td>8.0</td>
<td>6.8</td>
<td>100</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Concentration with PEG</td>
<td>50</td>
<td>10.2</td>
<td>8.8</td>
<td>80</td>
<td>6.7</td>
<td>6.2</td>
</tr>
<tr>
<td>Steric chromatography with CPG column</td>
<td>75</td>
<td>10.1</td>
<td>10.0</td>
<td>80</td>
<td>6.2</td>
<td>6.1</td>
</tr>
<tr>
<td>Linear gradient with amido trizote</td>
<td>10</td>
<td>10.8</td>
<td>10.8</td>
<td>75</td>
<td>5.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Steric chromatography with CPG column</td>
<td>2</td>
<td>11.2</td>
<td>11.0</td>
<td>25</td>
<td>4.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Steric chromatography with CPG column</td>
<td>3</td>
<td>11.0</td>
<td>11.0</td>
<td>25</td>
<td>4.3</td>
<td>4.8</td>
</tr>
</tbody>
</table>

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**Fig. 2. Buoyant density centrifugation of LCM virus in a linear amido trizote gradient.** Uninfected L-cell cultures were grown in the presence of either [3H]uridine or 14C-labeled amino acids. Disrupted cells plus PEG-concentrated medium were mixed with culture fluid from virus-infected L-cell cultures, and infectious virus was purified as described in the text and schematically represented in Fig. 1. The material from the 1.25 g/cm2 amido trizote cushion was centrifuged to equilibrium in an SW41 rotor at 40,000 rpm for 16 h. Fractions were taken by collecting drops from the pierced bottom of the tube. Symbols: ○, infectivity; △, 3H radioactivity; ▲, 14C radioactivity; □, density.
of the virus, which is rapidly lost as soon as proteins in the menstruum have dropped below a certain threshold level (13). During our work with this virus and its interaction with Mus musculus, we had come to the realization that condition sine qua non for further progress was a precise knowledge of the virus' chemical and antigenic composition, and despite the discouraging results of others the task was taken up anew.

The first step, namely, concentration of the virus from cell culture medium, turned out to be relatively easy. At this stage, co-precipitated proteins still exert their protective effects, and phase exclusion of macromolecules by PEG, as well as precipitation with zinc acetate (28), leads to satisfactory results. It should be stressed that precipitates obtained with PEG 40,000, which is used by us, contain less nonviral proteins than the precipitates formed upon addition of PEG 6,000, even though the latter is more widely used to concentrate viruses (1, 26).

Purification without loss of infectivity turned out to be the major obstacle, and we might have failed had the method of chromatography on glass of controlled pore size (10) not been available. Its greatest advantage is rapidity, which allows runs to be completed within minutes. Thus, with columns of the sizes we routinely used this step takes less than 10 min, and 100 ml of concentrate can be purified on a column 2.5 by 150.0 cm within 15 min. Even with a thermal lability as great as the one characterizing our virus, this is a short time and losses due to heat inactivation are negligible. High-speed chromatography combined with large samples relative to column size reduce dilution effects and do not affect resolution if the virus remains in the exclusion volume. A further advantage of this method is that chromatography is possible with highly concentrated materials. Indeed, PEG-treated virus can be applied to the column without prior dissolution of the aggregates. The compound quickly penetrates into the glass pores, whereby its concentration is reduced, causing dissolution of the precipitate. The same effects cannot be obtained with gels which shrink because of the high osmotic pressure of the applied material.

Zonal centrifugation in sucrose gradients is possible but leads to dilution of the virus. In contrast, isopycnic banding results in concentration. However, centrifugation to equilibrium in density gradients employing materials which have proved their usefulness with numerous other viruses led to inactivation of 99% and more of the initial infectivity. This statement is especially true with cesium chloride but also applies to other compounds, namely, cesium-acetate, CsSO₄, RbCl, KBr, KI, potassium-tartrate, NaBr, NaI, LiBr, and LiI. The difficulty was overcome when we began using amido trizooate for the gradients. Because of its high absorbancy this material is widely used in clinical medicine to mark body cavities for X-irradiation. Our assumption that nonaggressiveness as observed with respect to human tissues would also apply to LCM virus, as naive as this may seem, turned out to be correct, and amido trizooate is now successfully employed in this laboratory to centrifuge LCM virus as well as other viruses (manuscript in preparation).

It should be noted that the density of the infectious virus as observed with this substance is only 1.14 g/cm³ rather than 1.18 g/cm³, as determined with sucrose (19). Probably our value comes nearer to the truth because sucrose is believed to dehydrate viruses, thereby increasing their apparent densities.

The virus obtained with the PEG procedure (Fig. 1) must be regarded as highly purified. Knowing that titration in cell cultures underestimates the actual number of infectious units by a factor of almost 10 (9; Popescu and Lehmann-Grube, submitted for publication) and assuming that, as is the case with all other viruses, not each physical particle is infectious, a specific infectivity of 10¹⁴ PFU/mg of protein can only be explained if the degree of purity is close to 100%. The values for purification, as they were
calculated from admixing isotopically labeled cell culture constituents before purification, probably grossly exaggerate the degree of residual contamination, as has already been stressed.

With our intention to characterize the LCM virus we do not claim priority. In several publications, Pedersen has presented valuable data (19–22) and Rawls and his associates have described the composition of the related Pichinde virus (2–4, 24, 25). However, a cautious attitude seems appropriate. The infectious virology of LCM virus (or indeed of all other arenaviruses) has not yet been unequivocally identified. That the structures, seen by electron microscopy in abundance, budding from the plasma membranes of infected cells (17) are virus specific, no one can deny. Whether the same structures, all or in part, are infectious is an open question. In our opinion, biochemical studies of the infectious virus require purification as the first step in order to avoid characterization of structures which are virus specific but not infectious.

ACKNOWLEDGMENTS

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LITERATURE CITED