A SIMPLE AND EFFICIENT METHOD FOR PURIFICATION OF INFECTIOUS RECOMBINANT ADENOVIRUS

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SUMMARY: Recently, the adenovirus expression vector attracts much attention for the application to gene therapy and the method to purify and concentrate adenovirus without loss of infectivity has become very important, especially for animal experiments and gene therapy of humans. In this report, we show a simple and efficient method for purifying infectious adenovirus. The method consists of sequential centrifugation in CsCl step gradients without loss of infectivity and can be completed in one day. The method maintained the viral infectivity after 10-fold concentration and seemed to remove more than 99.9% of carried-over proteins. We showed also that the buffers for dialyzing the purified virions influenced the stability of infectivity. The buffers of 10 mM HEPES - 1 mM EDTA - 10% glycerol and PBS (-) - 10% glycerol resulted in higher stability than did 10 mM HEPES - 1 mM MgCl2 - 10% glycerol. The method is may be useful in many applications of recombinant adenovirus.

INTRODUCTION

The application of replication-deficient recombinant adenoviruses began in the field of experimental gene therapy of human diseases, such as cystic fibrosis (1,2). Because this vector can accept a large size of foreign gene (up to 7.5 kb) and...
can infect either non-dividing or resting cells, a wide variety of applications can be expected in various fields such as in central nervous system (3), muscle (4) and liver (5) tissues. Moreover, this vector is a possible candidate for the live vaccine (6); thus, a method to purify and concentrate recombinant adenoviruses becomes important. Although the retrovirus vector has been used for ex vivo gene therapy, it is known to be rather difficult to concentrate this vector without loss of infectivity (7).

We have recently developed an efficient method to construct recombinant adenoviruses (S. Miyake et al., in preparation). Consequently, it is now expected that many researchers will soon need in vivo experiments, that is, inoculation of the recombinant adenoviruses into animals. Before the inoculation, the recombinant virus should be purified and concentrated. However, the established methods (8,9) to purify adenovirus virions in a buoyant CsCl density-gradient often cause a loss of the infectivity. The present report describes a simple and efficient method to purify adenovirus without loss of the infectivity with CsCl step gradients, a modified step gradient method to purify bacteriophage lambda (10).

**MATERIALS AND METHODS**

**Virus and cells:** The replication-defective recombinant adenovirus (Adex1SRLacZL) was human adenovirus type 5 (Ad5) lacking the E1A, E1B and E3 regions and bearing E. coli-LacZ expression unit at the E1 region. The detailed procedure for constructing this recombinant virus will be published elsewhere (S. Miyake et al., in preparation). The LacZ gene was inserted between the SRα promoter (11) and SV40 polyadenylation signal. The 293 cells (12), a human embryo kidney cell line transformed by Ad5 E1A and E1B genes, support propagation of replication-deficient recombinant adenoviruses. They were maintained in Dulbecco's modified minimum essential medium (DME) supplemented with 10% fetal calf serum (FCS). Briefly, the recombinant adenovirus was generated by in vivo homologous recombination in 293 cells between the transfer cassette (pAdex1SRLacZL) and EcoT22I-digested Ad5-dIX DNA (13) tagging with viral terminal proteins. Transfection was performed by the calcium phosphate method. To prepare the conventional adenovirus stock, a Bioruptor 200 (CosmoBio, Tokyo) was used for disrupting the infected 293 cells. Such a sealed-type sonicator is necessary for both safety and preventing from contamination of microorganisms.

**Titration:** The infectivity of recombinant viruses, or titers of virus stocks were determined by the plaque assay on 293 cells or an end-point cytopathic effect
assay (14) with following modifications. Fifty microliters of DME supplemented with 5% FCS (5%-FCS DME) was dispensed into each well of a 96-well tissue-culture plate, and then eight rows of three-fold serial dilution of the virus starting from 10⁻⁴ dilution were prepared. Then, 3 × 10⁵ of 293 cells in 50 μl of 5%-FCS DME was added to each well. The plate was incubated at 37 C in 5% CO₂ in air, and 50 μl of 10%-FCS DME was added to each well every three days. Twelve days later, the end point of the cytopathic effect was determined by microscopy, and 50% tissue culture infectious dose (TCID₅₀) was calculated. In our hands, one TCID₅₀/ml approximately corresponds to one plaque forming unit (PFU)/ml.

Staining: To detect the coexisting β-galactosidase activity in the virus stocks, a 5-μl sample was mixed with 35 μl of PBS(−) and the mixture incubated with 1 μl of a solution of 4M 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for 1 hr at 37 C and checked for the blue color developed.

RESULTS

Procedure for Purifying Adenovirus

A simple method to purify and concentrate infectious recombinant adenovirus was established and summarized in Fig. 1. AdexISRLacZL was infected to 293 cells at a multiplicity of infection of 20 PFU/cell. The cells and media were harvested three days after infection and the virus was released by four cycles of 30-sec sonication with a sealed-type sonicator Bioruptor 200 at its maximum power. Cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4 C. The supernatant (denoted as conventional virus stock) was overlaid on the first CsCl discontinuous gradient, which was centrifuged at 25,000 rpm for 2 hr at 4 C in an SW28 rotor (Beckman). The gradient was made up of 10 ml of 2.2 M CsCl in 10 mM HEPES (pH 7.4) and 5 ml of 4M CsCl in 10 mM HEPES (pH 7.4). The visible virus band was collected and mixed with an equal volume of a saturated CsCl solution in 10 mM HEPES (pH 7.4). This mixture was transferred to a new SW41 tube, and then a step gradient made up of 3 ml of 4M CsCl in 10 mM HEPES (pH 7.4) and 4 ml of 2.2 M CsCl in 10 mM HEPES (pH 7.4) was overlaid on the mixture. Then the tubes were centrifuged at 35,000 rpm for 3 hr at 4 C in an SW41 rotor (Beckman). The sharp band consisted of virus particles to be collected; a broad band might appeared above the sharp virus band due to proteins derived from cell lysate or, perhaps, empty virions, a virus shells without the genome DNA.
A simple method for purification of recombinant adenovirus

virus infection to 293 cells

virus propagation for 3 days at 37 C

harvest

sonication

centrifugation (10k rpm, 10 min, 4 C)

collection of the virus solution (conventional stock)

1st CsCl step gradient centrifugation (25k rpm, 2 hr, 4 C, SW28 rotor)

collection of the virus band

Addition of an equal volume of saturated CsCl

2nd CsCl step gradient centrifugation (35k rpm, 3 hr, 4 C, SW41 rotor)

collection of the virus band

dialysis against PBS-10% glycerol overnight

Fig. 1. A flow chart of the methods to purify and concentrate infectious adenovirus. The positions of virus bands after first and second step gradients are shown with horizontal arrows, and vertical arrows show the migrating directions of the virus particles.
Table I. Titers of purified virus stocks after dialysis

<table>
<thead>
<tr>
<th>Dialysis buffer</th>
<th>Titer (PFU/ml)</th>
<th>Apparent recovery(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-MgCl₂</td>
<td>6 × 10¹⁰</td>
<td>150</td>
</tr>
<tr>
<td>HEPES-EDTA</td>
<td>4 × 10¹⁰</td>
<td>100</td>
</tr>
<tr>
<td>PBS(−)</td>
<td>7 × 10¹⁰</td>
<td>170</td>
</tr>
</tbody>
</table>

1) HEPES-MgCl₂, 10% glycerol-10 mM HEPES (pH 7.4)-1 mM MgCl₂; HEPES-EDTA, 10% glycerol-10 mM HEPES (pH 7.4)-1 mM EDTA; and PBS(−), 10% glycerol-PBS(−).

2) The original titer of the viral stock before purification was 2 × 10⁹ PFU/ml and recovered in 1/20 of the original volume after the purification. Thus, a titer of 4 × 10¹⁰ PFU/ml should correspond to 100% recovery.

To examine the stability of the purified virus in various dialysis buffers, the same purified virus solution was divided into three parts, and each dialyzed against (i) 10 mM HEPES, 1 mM MgCl₂, 10% glycerol (HEPES-MgCl₂ buffer), (ii) 10 mM HEPES, 1 mM EDTA, 10% glycerol (HEPES-EDTA buffer) and (iii) Ca²⁺, Mg²⁺-free Dulbecco’s PBS [PBS (−)] with 10% glycerol [PBS (−) buffer]. Two hours later, each buffer was changed to fresh one and dialysis was continued overnight. Each sample and the original virus stock prepared by the conventional procedure were titrated with 293 cells (Table I). The titers of purified virus stocks reached 4 to 7 × 10¹⁰ PFU/ml. The purified virus dialyzed against either HEPES-EDTA or PBS (−) buffer showed a much higher titer than did that dialyzed against HEPES-MgCl₂ buffer (Table I). The observed infectivity of purified virus was equal to or, unexpectedly, higher than the calculated one irrespective of dialysis buffers examined (see Table I, footnote 2). The fact that the purified virus titer exceeded the theoretical 100% may have been due to some factor(s) inhibiting virus infection had been removed during purification.

Elimination of Carried-over Proteins

The β-galactosidase expressed by the recombinant adenovirus was present in the virus stock together with all of the cellular and serum proteins. To examine if coexisting proteins had been removed by the purification, the β-galactosidase activity was measured in the purified and conventional virus stocks.
(Table II). Each sample was 10-fold serially diluted with PBS (−) and each was stained with X-gal. In the original virus stock the activity of β-galactosidase was detected down to a 100-fold dilution; whereas a low activity was detected only in the undiluted sample of the purified virus stock. Since the purified virus had a roughly 10 times higher titer than the original virus stock, the result suggests that the amount of β-galactosidase derived from 293 cells present in the purified virus stock decreased to 0.1%.

### Stability of Purified Virus

To determine stability of the purified virus, each sample of the purified virus stocks after dialysis was subjected to either one cycle of freezing-and-thawing or two cycles of 30-sec sonication (Fig. 2). More than 40% of the infectivity of that of the stock before purification was maintained after the freezing-and-thawing, when either HEPES-EDTA buffer or PBS (−) buffer was used. When the HEPES-MgCl₂ buffer was used, however, the titer reduced to 19% of the original infectivity. Similarly, while virus titers after the sonication in HEPES-EDTA buffer or PBS (−) buffer retained at least 20% of the infectivity; the titer dropped to 7.5% in HEPES-MgCl₂ buffer. The results suggest that the contents of the stock solution are important for maintaining the infectivity after sonication and freezing-and-thawing and that the purified virus is more stable in HEPES-EDTA buffer or in PBS (−) buffer than in HEPES-MgCl₂ buffer.

### Table II. β-Galactosidase activity contaminating in the virus stocks

<table>
<thead>
<tr>
<th></th>
<th>Virus titer (PFU/ml)</th>
<th>β-galactosidase activity at a virus dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁰</td>
</tr>
<tr>
<td>Conventional stock ¹</td>
<td>2 × 10⁹</td>
<td>+++</td>
</tr>
<tr>
<td>Purified stock ²</td>
<td>4 × 10¹⁰</td>
<td>±</td>
</tr>
</tbody>
</table>

¹) Virus stock before purification.
²) Dialyzed against HEPES-EDTA buffer.
DISCUSSION

The titer of the conventional adenovirus stock often reaches $10^9$ PFU/ml and therefore is usually enough for in vitro experiments without further concentration. Of course, however, the expression of the desired gene essentially increases 10-fold when a 10-fold concentrated virus stock is used. To apply recombinant adenoviruses to in vivo experiments, the steps of purification and concentration are absolutely necessary because: (i) the volume of the inoculum is often limited, (ii) it is difficult to distinguish whether the observed effect is due to the desired in vivo-expressed protein or due to the carry-over of the protein produced during viral stock preparation in 293 cells, and (iii) human cellular proteins present in the conventional stock can induce various immune responses in the inoculated animals.
The previously-established method (8,9) to purify adenovirus virions consists of the following steps: (i) concentration of virus on "CsCl cushion", (ii) two cycles of buoyant CsCl density-gradient centrifugation in 10-mM Tris-HCl (pH 8.0) buffer, and (iii) dialysis against the Tris-HCl buffer above. The method has been frequently used for purification and characterization of the viral genome and structural proteins, since a large amount of infected cells can be processed by this method. In our hand, however, this method yielded a virus stock with a markedly reduced infectivity. The reason for loss of infectivity is not clear but appears partly over-concentration of virions, followed by aggregation at the dialysis and storage steps.

The method described here is based essentially on the purification procedure of bacteriophage lambda. Essential modification of the conditions was the density of two cycles of CsCl step gradients from 3M/5M for lambda phage to 2.2M/4M for adenovirus, in response to their particle density. The method with a step gradient described in this report appeared to have several advantages over with the previous method that uses buoyant gradient. (i) This method is easier and quicker. Although the previous method needs two or three days for the whole procedure, this method takes only one day; the quick procedure may eventually reduce loss of infectivity. (ii) The step gradient possibly prevents overconcentrating virions which may cause aggregation and loss of infectivity. (iii) The virus purity after two cycles of step gradients may be higher than that after two cycles of buoyant gradients, because separation of the target molecule was maximized by optimizing the CsCl densities of the steps. Efficient elimination of human cellular proteins is important for quality control for gene therapy.

We examined also the effects of dialysis buffers on the stability of the purified virus, because purified and concentrated adenovirus often loses its infectivity by self-aggregation during dialysis and storage at -80 C. Another important reason is that the purified virus stock is often used for inoculation to animals and hence PBS is usually preferred for this purpose. We showed that the virus infectivity was maintained in all three buffers containing 10% glycerol. Glycerol was added mainly to stabilize the virus during storage at -80 C and could be omitted if used immediately after dialysis. HEPES buffer is used because of its stronger buffer action and safety for animal cells than Tris buffer. We recommend HEPES-EDTA-glycerol buffer as well as the PBS-glycerol buffer. Rosenfeld et al. (1) used Tris-MgCl₂-glycerol buffer similar to our HEPES-MgCl₂-glycerol buffer, but this buffer showed only limited stability after either freeze-thaw or sonication: therefore, we do not recommend this buffer system.
Finally, this simple method for purification removed 99.9% of the carried-over β-galactosidase expressed during viral propagation in 293 cells. It seems reasonable to expect that human cellular proteins of 293 cells and bovine serum proteins in the culture medium were similarly removed. This point is, of course, important for avoiding undesirable immune responses occurring in the inoculated animals.

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REFERENCES


