Large-Scale Purification of Japanese Encephalitis Virus from Infected Mouse Brain for Preparation of Vaccine

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Infectious mouse brain is still a common source of Japanese encephalitis (JE) virus vaccine and diagnostic viral antigen because of the advantage of a high virus yield. However, with respect to the purity of the starting materials, infectious mouse brain preparation is inferior when compared with tissue culture-propagated materials.

Various procedures for separation of brain components from viral suspension have been described, including protamine sulfate treatment (1, 2), fluoro carbon treatment (15), aluminum phosphate gel chromatography (13), ethanol precipitation (11), and sucrose gradient centrifugation (12). In spite of such studies, difficulty in minimizing the amount of host protein still remains in large-scale purification procedures. Previous authors (4–8, 10) showed that precipitation concentrates purify several enveloped animal viruses without loss of infectivity. We have applied this method to mass purification of JE virus as a source of vaccine or diagnostic antigen.

MATERIALS AND METHODS

Virus. JE virus, Nakayama NIH strain, was kindly supplied by X. Oya, National Institute of Health, Tokyo, Japan, and passed through suckling mice by the intracerebral route for 38 generations in our laboratory.

Mice. Twenty-one- to twenty-eight-day-old ddY strain (randomly bred) mice were used for the antigenicity assay, and mice weighing 10 to 12 g were used for preparation of crude viral materials.

Buffered saline. Phosphate-buffered saline (1/100 M, pH 7.8) was employed.

Chemicals. Polyethylene glycol (PEG) 6,000 was purchased from Wako Pure Chemicals Industries, Osaka, Japan. Protamine sulfate was purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan.

Inactivation of virus. The samples were inactivated with 0.04% Formalin for about 30 days at 4°C.

Zonal centrifugation. The zonal centrifuge consisted of a K-II rotor driven in a K-II zonal ultracentrifuge manufactured by Electro-Nucleonics Inc., Fairfield, N. J.

The sucrose gradient solution was formed by reorientation and self-formation with a static loading of 16,000 ml of phosphate-buffered saline and 16,000 ml of 60% sucrose-buffered saline.

The operating speed was 35,000 rpm. The flow rate of the samples was kept at 100 ml/min.

Assay of virus infectivity. The infectivity of JE virus was assayed by plaque formation on chicken embryo fibroblast cells (14).

Assay of hemagglutinin. The hemagglutinin was titrated with goose erythrocytes at pH 6.4 as described by Clarke and Casals (3).

Protein determination. Protein content was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard.

PEG determination. PEG was determined by the procedure of Stevenson (16) after treatment of sample with acetone for removal of protein. The limit of detection is 0.002 mg/ml.

Hemoglobin determination. Hemoglobin was determined by the hemoglobin cyanide method (17). The amount of hemoglobin in the range below the limit of detection by the hemoglobin cyanide method was computed from the iron content determined by atomic absorption spectroscopy (18).

PF. Purification factor (PF) was calculated by dividing the specific activity (plaque-forming units per milligram of protein) of a given specimen after purification of the initial materials by specific activity.

Immunogenicity test. Groups of 10 mice were inoculated intraperitoneally with either a standard reference vaccine or PEG-purified vaccine two times at weekly intervals. Each mouse was given 0.5 ml of vaccine diluted 32-fold each time.

Blood samples were collected from each mouse on day 15 after the first inoculation. The serum was collected and inactivated for 30 min at 56°C, and titrated for JE antibody by the neutralization test with the plaque reduction method (18). Serum neutralization titers represented the reciprocal of the highest

54
dilution showing 50% reduction of the control plaque number.

RESULTS

Purification of virus. A 20% suspension of JE virus-infected mouse brain was prepared with phosphate-buffered saline.

This suspension was treated with protamine sulfate at a final concentration of 1 mg/ml, placed in a cold room for 3 h to form flocules, and then centrifuged for 30 min at 16,000 × g. The supernatant fluid was used as the starting material in the experiments described here.

A 30-ml amount of the supernatant fluid was mixed with an equal volume of PEG solution at various concentrations, and the mixtures were centrifuged for 30 min at 16,000 × g.

The supernatant fluid was decanted, and precipitates were resuspended with 30 ml of phosphate-buffered saline. The infectious titers, protein contents, and hemoglobin contents in both the supernatant and pellet were assayed (Fig. 1).

Most of the infectious virus particles were recovered in the pellets at a final concentration of 4% or more PEG.

At this concentration of PEG, the protein content in the pellet was decreased to about 1/4 of the initial protein content. (PF = 4) The content of hemoglobin in the pellet was decreased to less than 0.003 mg/ml.

Subsequent experiments were performed at 4% PEG as a final concentration.

Removal of PEG and further purification. The resuspended pellet precipitated with 4% PEG was treated with ethanol for removal of residual PEG and for further purification.

A 30-ml amount of the resuspended pellet was mixed with an equal volume of various concentrations of ethanol and incubated overnight at 4°C. After centrifugation of the mixtures for 30 min at 16,000 × g, the supernatant fluid and pellet were assayed for infectious titers, protein contents, and PEG residues (Fig. 2). Figure 2 shows that about 100% of the infectious virus particles were precipitated, and the protein content in the pellet was decreased to about 1/14 of the initial content at a final concentration of 8% ethanol (PF = 14).

In addition, PEG contamination in the pellet was decreased to less than 0.002 mg/ml.

Purification of large volumes of viral suspension. Subsequently, 5,000 ml of viral suspension was purified under optimum conditions obtained from Fig. 1 or 2 (1 mg of protamine sulfate per ml, 4% PEG, and 8% ethanol at the final concentration).

The results are shown in Table 1. Purification efficiency on a large scale was about the same as that on a small scale.

Application of ultracentrifugation to the removal of PEG. The technique of zonal centrifugation was applied to the removal of residual PEG. The procedure of zonal ultracentrifugation was as described in Materials and Meth-

![Fig. 1. Precipitation of JE virus by PEG. Original viral suspension contained 10^8.1 plaque-forming units (PFU) of infectivity per ml, 0.325 mg of protein per ml, and 0.270 mg of hemoglobin per ml. Symbols: ●, PFU in pellet; △, protein content in pellet; ○, hemoglobin content in pellet.](image1)

![Fig. 2. Removal of PEG and further purification of JE virus suspension by ethanol precipitation. Original viral suspension contains 10^8.5 plaque-forming units (PFU) of infectivity per ml, 0.181 mg of protein per ml, and 0.225 mg of polyethylene glycol per ml. Symbols: ●, PFU in pellet; △, protein content in pellet; ○, PEG content in pellet.](image2)
ods. Ten liters of viral suspension clarified with protamine sulfate was processed in the K-II rotor at a flow rate of 100 ml/min and a maximum speed of 35,000 rpm. The profiles of the resulting sucrose gradient are shown in Fig. 3a. The viruses banded at a sucrose concentration of 43% (density = 1.20 g/cm³). The protein peak banded at a sucrose concentration of 28% and coincided well with the hemoglobin peak.

When the viral suspension was treated with PEG before centrifugation, the total amount of protein was reduced, and the protein peak moved nearer to the peak of viral infectivity. (Fig. 3b) In this purification process, PEG contamination remained in each fraction.

Antibody response. Six lots of vaccine purified by this method were compared for their abilities to induce neutralizing antibodies (Table 2). For the comparison, standard reference vaccine of the National Institute of Health (Japan) was tested.

The PEG-purified vaccines induced a good neutralizing antibody response in mice.

**Table 1. Purification of a large volume of JE virus**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infectivity (PFU/ml, log₁₀)</th>
<th>Protein (mg/ml)</th>
<th>Hemoglobin (mg/ml)</th>
<th>PEG (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protamine sulfate</td>
<td>8.5</td>
<td>0.875</td>
<td>0.290</td>
<td></td>
</tr>
<tr>
<td>Protamine sulfate-PEG</td>
<td>8.4</td>
<td>0.119</td>
<td>&lt;0.003</td>
<td>0.138</td>
</tr>
<tr>
<td>Protamine sulfate-PEG-ethanol</td>
<td>8.3</td>
<td>0.013</td>
<td>&lt;0.003</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

*PFU, Plaque-forming units.*

**Table 2. Antibody response of purified vaccine in mice**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Potency (log₁₀ neutralization indexes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purified vaccine&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>3.3</td>
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<tr>
<td>5</td>
<td>3.2</td>
</tr>
<tr>
<td>6</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Purified vaccines contain from 0.013 to 0.016 mg of protein per ml and from 10<sup>-7</sup> to 10<sup>-9</sup> plaque-forming units of preinactivation infectivity per ml.

<sup>b</sup> Standard reference vaccine of National Institute of Health of Japan.

**DISCUSSION**

Since the precipitating effect of PEG on virus was first observed by Hebert (4), many authors (5-8, 10) have applied this method to many kinds of enveloped animal viruses for concentration and purification.

In this study, we extended the application of PEG as the virus precipitant to purification of large volumes of JE virus grown in mouse brain for vaccine or diagnostic use.

The viruses could be precipitated at PEG concentrations of 4% or higher (Fig. 1). The recovery of infectivity in pellets was about 100%, whereas 70% or more of the host protein was left behind in the supernatant fraction.

It is better to use 4% PEG as a final concentration, since the use of low concentrations has the advantage that the host protein remains in

![Fig. 3. Profiles of continuous-flow isopycnic banding of JE virus in sucrose gradients. The samples were clarified with protamine sulfate (a) and with protamine sulfate and PEG (b).](http://aem.asm.org/)
the supernatant.

In mouse brain suspensions, a large amount of hemoglobin is present (Table 1), and some continues to remain throughout the various purification processes, especially in large-scale purification. In some commercial vaccines, about 30% of the total protein content is protein originating from hemoglobin. Hemoglobin could be separated from JE virus suspension by PEG precipitation. (Fig. 1 and 3a and b)

In the present experiments, protamine sulfate treatment of brain suspension was performed before PEG precipitation to prevent the loss of infectivity in the process, but this may not always be necessary if the appropriate conditions for treatment, e.g., concentration of brain suspension and time of incubation, are determined.

Purification of the concentrate from the remaining PEG was achieved by precipitation with ethanol (Fig. 2). The optimum concentration of ethanol was 8 to 12%. At this concentration of ethanol, the recovery of infectivity was about 100%, and PEG contamination in the viral suspension was decreased to less than 0.002 mg/ml, although it is difficult to remove the PEG by zonal ultracentrifugation.

The viral suspension purified by the method mentioned above was sufficient for use as a vaccine source with respect to both the antibody response and purity from the host protein.

ACKNOWLEDGMENTS

We thank Isao Yotsuya, Nobutoshi Yamazaki, and Masatoshi Hayashi for their technical assistance.

LITERATURE CITED