PURIFICATION AND ANALYSIS OF 17DD YELLOW FEVER VACCINE VIRUS

ABSTRACT

A methodology to concentrate and purify the vaccinal 17DD Yellow Fever virus (17DD YFV), produced in chick embryos, was developed in this work, enabling the determination of some of their characteristics. Alternative diluents were tested for the extraction of 17DD YFV from infected chick embryo tissues, regularly used for the vaccine production. The use of a buffer containing Tris, ethylenediaminetetraacetic acid (EDTA), and cytokine, was found to increase the yield of virus extraction relative to medium 199, which is normally used as a diluent in the vaccine production. Purified virions were obtained after protamine treatment, polyethyleneglycol (PEG) precipitation, and fractionation in sucrose density gradients by ultracentrifugation. A virus band was well separated from the majority of contaminating proteins, which remained in the low-density fractions. The presence of virions, determined by plaque forming unit (PFU), and hemagglutination (HA) of purified 17DD particles were found at a density of 1.164 g/ml. The ratio of viral particles/PFU demonstrated that for each infectious unit there were at least 223 non-infectious units in the purified vaccine preparation.
INTRODUCTION

The Yellow Fever viruses (YFV) are classified in the Flavivirus group of the Flaviviridae family. Yellow fever (YF) infections in humans have caused thousands of deaths during the evolution of western civilization. In urban areas it is transmitted by infected Aedes aegypti in a mosquito-man-mosquito cycle, but YF is endemic in sylvan areas of Central and South Americas, and Africa, with participation of monkeys and mosquitoes in the transmission cycles (Strode, 1951). The current urban widespread distribution of Aedes aegypti in combination with the impossibility of interrupting the sylvan cycle of YF present a continuing threat to non-immune human populations in certain urban areas.

In 1937, Theiler and Smith derived an attenuated variant of Yellow Fever virus from the virulent Asibi strain. Attenuation was achieved by serial passage in Rhesus monkeys, followed by serial passages in the embryonic mice tissue cultures, whole chicks, and chicks with taken nervous systems. This historical protocol generated a virus strain designated 17D that was associated with fully attenuated infections in humans inducing long-term immunity (Theiler & Smith, 1937a; Theiler & Smith, 1937b). Production of the vaccine was established in Brazil in 1937 and a seed lot system was introduced few years later to limit the number of serial passages and thereby reduce the risk of over-attenuation or reversion to virulence (Post et al., 2001). Since that time, FIOCRUZ, Brazil has produced the vaccinal strain of 17DD YFV at a constant passage level in chick embryos.

Very limited data on the antigenic and physicochemical properties of 17DD YFV are available. Since a previous purification methodology showed relatively low 17DD-virus recovery from chick embryo homogenates (Post et al., 1991), a purification technique previously developed for Alphaviruses (Cabral, 1986; Villela, 1990; Villela et al., 1994) was tested and a preliminary characterization of the purified virions was carried out.

We also hypothesized that calcium ions present in chicken embryo tissues would contribute to the aggregation of cellular and viral membranes (Darnell et al., 1990; Stryer, 1988), thereby pelleting virions during centrifugation steps required in the concentration and purification methodologies described here. In order to test our hypothesis, we verified whether the homogenization of 17DD-infected chicken embryo tissues with calcium chelator-containing solutions would increase the recovery of vaccinal virus particles in these methodologies.

MATERIAL AND METHODS

Yellow Fever Virus (YFV): the 17DD YFV strain was obtained from infected chick embryo homogenates regularly used to produce the YF vaccine at Instituto Oswaldo Cruz, Biomanguinhos, Brazil.

Current process of YF vaccine production at FIOCRUZ: Theiler and Smith first established
the protocol to produce the 17DD YF vaccine (Theiler & Smith, 1937b) that received few modifications after its implementation in Brazil (Post, 1996). Briefly, the seed virus is inoculated into 9-day old chick embryos. After a 3-day incubation, 44 live embryos are collected from the eggs and triturated in the presence of 50 ml of ultra pure water. The homogenate, or embryonic pulp, is centrifuged and the supernatant is collected and frozen at -70°C, to await the results of sterility and infectivity tests. Approved homogenates are then thawed and diluted out with medium 199 and thermal stabilizer solution (TSS) to reach the vaccinal infectious titer. The final vaccine is prepared by distribution into vials and freeze-drying.

**Solutions:** A) medium 199 (Life Technologies Inc. Grand Island, NY). B) Thermal stabilizer solution (TSS): medium 199 (34% v/v), sucrose (12% w/v), and monosodium glutamate (5% w/v) in H2O. C) TSS-C: sodium citrate 12.5mM in TSS. D) TEC: tris/HCl 0.08M, EDTA 0.02M, cysteine 0.02% (w/v), and NaCl 0.5% (w/v) in H2O, pH 8.4.

**Recovery of 17DD virus from infected chick embryos:** 5 groups of 10 infected chick embryos from a single vaccine production lot were aseptically harvested and separately weighed. Solutions A, B, C, and D were each added to one group, in a volume corresponding to 35% of the total weight (w/v) of the embryos per group. One group did not receive any solution and was used as a control. All groups were immediately homogenized at 12000 rpm in ice bath using a tissue blender and the pulps were centrifuged at 7000g for 30 min at 5°C. Supernatants were collected and stored at -70°C pending determination of 17DD infectious titer by PFU, using Vero cells, in each supernatant.

**Virus concentration:** A 17DD-infected embryo pulp obtained from the production of YF vaccine was thawed and diluted 1:5 in D solution above (TEC). The pulp-TEC mixture was re-homogenized for one minute in an ice bath and centrifuged at 7000g for 30 min. Supernatant (SUP 0) was further clarified by incubation with protamine sulfate (PS) at a final concentration of 0.9 mg/ml in an ice bath for 30 min. After centrifugation as above, viruses in the supernatant (SUP 1) were precipitated by addition of polyethylene glycol 6000 Kd (PEG 6000 8% w/v), during an overnight incubation at 4°C. Following a centrifugation at 1500g for 20 min at 5°C, the supernatant (SUP-PEG) was discarded and the pellet was resuspended on ice to 1/50th of the SUP1 volume, in TEC-containing 8% of sucrose (w/v). This concentrated virus preparation was clarified by centrifugation at 1500g for 10 min (SUP 2). The pellet was resuspended in an equal volume of TEC-sucrose 8%, generating the SUP 2r, which was combined with SUP 2. Aliquots of each step were tested for virus recovery determined by PFU using Vero cells.

**Virus purification:** seven ml of SUP 2 and SUP 2r mixture were layered on 26 ml of 15-50% (w/v) sucrose density gradients prepared in 35 ml ultracentrifuge tubes and centrifuged in a Sorvall OTD 65B (Kendro Laboratory Products, Newtown, CT) at 100,000g for 18 hours at 5°C. Following
ultracentrifugation, fractions of 1 ml were collected from the top of the tubes and stored at -70°C, pending analyses described below:

**Density:** was determined for all 33 fractions of the sucrose gradient from the refractive index, as measured at 25°C using an Abbe refractometer (Misco, Cleveland, OH) and converted to density using the conversion table of Weast and Astle (1980).

**Infectivity assay:** was conducted by determining the (PFU) in Vero cells as described by Lopes et al. (1987; 1988).

**Hemagglutinin activity:** was assayed by the method of Lennete and Shmidt (6) and adapted by Lopes et al. (1987; 1988) using goose erythrocytes. The optimum pH for hemagglutination of 17DD virus was experimentally determined by using buffers ranging from pH 5.75 to 7.40. All other HA assays were then conducted at pH 6.20.

**Protein concentration:** was determined from absorbency values at 260 nm and 280 nm in a Lambda 3A UV/VIS spectrophotometer (Perkin-Elmer Instruments, Shelton, CT).

**RESULTS**

**Recovery of 17DD virus from infected chick embryos:** Solutions A, B, C, and D were added in a volume corresponding to 35% of the weight of the 17DD-infected chick embryos, before the triturating step that generates the embryonic pulp. This volume was used in order to obtain a 1:2 dilution of the supernatants, after centrifugation of the pulp, in relationship to the control group of chick embryos that did not receive any solution. The rates of viral recovery were determined by comparing the PFU titer of supernatants from each pulp with the supernatant of the control group (Table 1).

We observed a 36% and 3.2% decrease in the infectious titers when medium 199 (solution A) and TSS (solution B) were used, respectively. In contrast, when TSS-citrate (solution C) and TEC (solution D) were used, we observed a 6.2% and 13.8% increase in the infectious titer, respectively. The use of TEC provided a recovery yield of virus 50% higher than the medium 199 normally used in vaccine production process.

Concentration and purification processes: Recoveries of virus at different steps of the concentration and purification processes are shown in Table 2. The TEC buffer was used in these methodologies because the comparative analysis of the diluents tested here demonstrated that TEC yielded the highest virus recovery (Table 1). The optimum concentration of PS was experimentally determined to be 0.9 mg/ml (data not shown). Lower concentrations of PS failed to give a clear supernatant following centrifugation, while higher levels generated pellets that were extremely difficult to dissolve following PEG precipitation. Loss of infectious virus was very small (7.3%) during this step of the process. PEG was highly effective in precipitating viruses (>99.6%) and recovery of infectious virus from the centrifuged pellet was satisfactory at 77.2%.

In the density gradient, the majority of infectious viruses were found in densities ranging from 1.158
to 1.171 g/ml, where we detected $14.3 \times 10^4$ PFU/ml. This band was well separated from the majority of contaminating proteins, which peaked at the density of 1.059 g/ml (Fig. 1).

In order to determine the pH that allowed the highest titer of hemagglutination (HA) mediated by 17DD viruses, we tested purified virions obtained from fractions of the gradient column with the highest titer in PFU, in a pH ranging from 5.75 to 7.4. The highest titer of HA, was observed at pH 6.2 (data not shown), which was used to test all fractions of the gradient for this activity. The peak of HA activity of 320 HAU/ml/10^6 cells was detected at the same density in which the highest PFU titer was observed (Fig. 1). Taking in consideration the number of cells used in the HA assay ($10^6$ goose erythrocytes), we calculated the presence of at least 320 $\times 10^5$ hemagglutinating particles in the purified preparation of vacinal virus (Clark & Casals, 1958; Lennete & Schmidt, 1979; Lopes et al., 1987). Therefore, the ratio of total particles/PFU in the purified preparation can be calculated by $320 \times 10^5 / 14.3 \times 10^4$ suggesting that for each infectious unit there are at least 223 non-infectious particles with fusogenic activity.

**DISCUSSION**

The comparative analysis of infectious titers of 17DD YFV from an undiluted chick embryo extract in relationship to those diluted by different solutions, demonstrated that solutions C (TSS-C) and D (TEC), which contain calcium chelator substances such as citrate and EDTA, respectively, could increase the recovery of infectious particles during the preparation of these extracts. In addition, our findings indicated that the use of medium 199 might have contributed to a loss of 49.8% of the viral particles produced in the chick embryo tissues, in relationship to TEC buffer. Therefore, in order to increase the productivity of the YF vaccine, which would also promote an economy of the vaccine seed lot, the inclusion of a solution containing a calcium chelator, such as EDTA, should be further evaluated.

The 77% recovery rate of the concentration methodology for 17DD viruses described here was comparable to the rate described for Alphaviruses (Cabral, 1986) and much higher than the 5% rate previously shown for 17DD viruses (Post et al., 1991). The differences in the recovery rates in these studies may be due to the chemical characteristics of the components of the TEC buffer used here. For example: I) the use of Tris contributes to the preservation of the integrity of the E and M glycoproteins of the Flavivirus envelope. This is achieved by maintaining the pH of the vaccine extract around 8.0, above the pH in which the fusion mechanism between viral and target membranes is triggered (Allison et al., 1995), defined here as pH 6.2 for the 17DD virus. II) EDTA has a calcium chelator property that may prevent the aggregation of negatively charged cellular or viral membranes by calcium ions (Darnell et al., 1990; Stryer, 1988), avoiding the loss of virus particles during centrifugation steps of the
methodologies. III) Cysteine: during the titration of chick embryos, intracellular cystine synthase may be released into the extract. The presence of this enzyme may contribute to the formation of disulfide bridges, aggregating cellular or viral membranes. The presence of soluble cysteine in the TEC buffer may play a role inhibiting the aggregation of membranes, also avoiding the loss of viral particles during the extraction process (Darnell et al., 1990).

The virus particles/PFU ratio suggested that for each 17DD infectious unit purified in this study there were at least 223 non-infectious other particles with fusogenic capability. The high ratio of non-infectious/infectious particles could be an exclusive characteristic of the 17DD preparation, contributing to the safety and high efficacy of the vaccine, by induction of interferon production in vaccine recipients (Bonnerie-Nielsen et al., 1995). This hypothesis is corroborated by the analysis of a 17D preparation that after passage in BHK or SW-13 cells decreased the ratio virus particles/PFU to 10 and lost the 17D vaccinal characteristic (Lindenbach & Rice, 1997). Moreover, the fusogenic ability of Flaviviruses is directly correlated to their infectivity, since when this fusion occurs on the plasma membrane of permissive cells, the viral genetic material is transferred into the cytoplasm, allowing the initiation of virus replication (Allison et al., 1995). Therefore, the high ratio of non-infectious/infectious particles described here, may be a true representation of the 17DD vaccine preparation and not a consequence of a loss of infectivity due to the methodologies employed.

Finally, we conclude that the virus concentration rate of 77.22% and the purification process, which effectively separated 17DD virions from less dense proteins from chick embryo cells, are satisfactory methodologies developed for 17DD virus. Consequently, we were able to determine: density, fusogenic pH-dependent profile, and rate of infectious/non-infectious viral particles present in the 17DD vaccine preparation, contributing to a better understanding of the characteristics of this vaccine, which is considered by the World Health Organization as the best vaccine available for human use.

REFERENCES


4. Clark, D.H.; Casals, J. 1958. Techniques for hemagglutination and hemagglutination-


Table 1: Effects of different solutions in the recovery of 17DD virus obtained from infected chick embryos.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Volume of supernatant (ml)</th>
<th>PFU/ml</th>
<th>PFU total</th>
<th>Gain or loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>36</td>
<td>3,162,000</td>
<td>113,832,000</td>
<td>0</td>
</tr>
<tr>
<td>Medium 199</td>
<td>72</td>
<td>1,011,840</td>
<td>72,852,480</td>
<td>-36%</td>
</tr>
<tr>
<td>TSS</td>
<td>72</td>
<td>1,530,408</td>
<td>110,189,376</td>
<td>-3.2%</td>
</tr>
<tr>
<td>TSS-C</td>
<td>72</td>
<td>1,679,022</td>
<td>120,889,584</td>
<td>+6.2%</td>
</tr>
<tr>
<td>TEC</td>
<td>72</td>
<td>1,799,178</td>
<td>129,540,816</td>
<td>+13.8%</td>
</tr>
</tbody>
</table>

Groups of ten 17DD-infected chicken embryos were separated and weighted. Volumes of each solution corresponding to 35% of embryos (w/v) were added to each group before the homogenization of embryos in a tissue blender. After centrifugation of the pulp, supernatants were collected and the total number of PFU was calculated by multiplying the volumes of the supernatants per infectious titer in PFU/ml. Logarithmic numbers were converted to whole numbers in order to calculate the recovery rates obtained by the use of different diluents.

Table 2: Recovery rates of each step of the concentration methodology.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Volume (ml)</th>
<th>Titer (PFU/ml)</th>
<th>Titer (total PFU)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUP 0</td>
<td>888</td>
<td>22,909</td>
<td>20,343,192</td>
<td>100.00</td>
</tr>
<tr>
<td>SUP 1</td>
<td>850</td>
<td>22,182</td>
<td>18,854,700</td>
<td>92.68</td>
</tr>
<tr>
<td>SUP-PEG</td>
<td>820</td>
<td>&lt;100</td>
<td>&lt;82,000</td>
<td>&lt;0.40</td>
</tr>
<tr>
<td>SUP 2 + SUP2r</td>
<td>35</td>
<td>448,828</td>
<td>15,709,012</td>
<td>77.22</td>
</tr>
</tbody>
</table>

Sup 0 was the initial step of the concentration methodology and was considered the basis for calculating the recovery rates of the remaining steps. Logarithmic numbers were converted to whole numbers in order to calculate the recovery rates. The total number of PFU was calculated by multiplying the volume of the supernatant per infectious titer in PFU/ml. Fractions 19, 20, and 21 of the density gradient, in which the peak of PFU was observed, were pooled to calculate the recovery rate of purified virus.
Figure 1: Analyses of density, protein concentration, and titers of PFU and HAU in fractions of density gradient column used for purification by ultracentrifugation of concentrated 17DD viral particles.