Vaccine 26 (2008) 3680–3689

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Long-term stability of Vero cell-derived inactivated Japanese encephalitis vaccine prepared using serum-free medium

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ARTICLE INFO

Article history:
Received 9 August 2007
Received in revised form 22 April 2008
Accepted 28 April 2008
Available online 19 May 2008

Keywords:
Japanese encephalitis virus
Serum-free medium
Stability

ABSTRACT

We established a method of producing a Vero cell-derived Japanese encephalitis vaccine using serum-free medium, and tested its stability using various stabilizers during the inactivation process and storage at 4 °C and 28 °C.

Similar to previously reported results of cell culture in serum-containing medium, Vero cells were cultured in a serum-free medium multiplied well, and the viral yield was successfully increased to about 10^9 PFU/ml. Following formalin-inactivation and purification via ethanol precipitation and sucrose density ultracentrifugation of the virus solution, the vaccine had the same quality as, and higher immunogenicity, the mouse brain-derived vaccine in current use. Testing of several stabilizers showed that the addition of 0.5% glycine during the virus inactivation process facilitated the maintenance of immunogenicity for a long period of time. Furthermore, the addition of 0.5% glycine and 1.0% sorbitol as vaccine stabilizers after purification led to the maintenance of immunogenicity for 1 year, not dependent on the storage temperature (4 °C or 28 °C).

These results indicate that, in contrast to the current mouse brain-derived vaccine, the Vero cell-derived vaccine can be prepared using serum-free medium containing no animal-derived components, and that the vaccine can be stored at room temperature by adding stabilizers, suggesting the possibility of producing room temperature-stable vaccines.

1. Introduction

Japanese encephalitis (JE) is a severe disease that is widespread throughout Asia, and is spreading beyond its traditional boundaries. Among the 35,000–50,000 annual cases of JE, about 10,000 are fatal, and a high proportion of survivors have serious neurological and psychiatric sequelae [1,2]. JE is caused by a mosquito-borne flavivirus called Japanese encephalitis virus (JEV) that belongs to the genus *Flavivirus* of the family *Flaviviridae*. In humans, JEV infection can cause severe central nervous system disorders including febrile headache, aseptic meningitis, and encephalitis. JEV is a small, enveloped virus consisting of a capsid [C], premembrane [preM], membrane [M], envelope [E], and nonstructural [NS] proteins [3]. The viral E protein, modified by glycosylation and dimerization during virion assembly, serves as the cell-receptor binding protein and the fusion protein for virus attachment and entry into the host. Antibodies directed against E protein neutralize the virus [4] and play an important role in protection [5].

There is no drug treatment for JE; therefore, vaccination is the single most important control measure. A vaccine produced by formalin-inactivation of JEV grown in mouse brains was first developed in the 1930s, and has been effective in reducing the incidence of JE in Japan since 1954. Although this vaccine is highly purified and safe, some theoretical risks remain: adventitious infectious agents and traces of impurities derived from the mouse brain may lead to adverse neurological events, such as acute disseminated encephalomyelitis (ADEM). To avoid such risks, a tissue culture-derived JE vaccine from Vero cells has been under development [6,7]. Vero cells derived from the African green monkey have been approved for viral vaccine production under specified regulatory guidelines [8,9], and are currently used for the production of rabies and polio vaccines. Moreover, Vero cells have been employed to produce bovine vesicular stomatitis virus, herpes simplex virus, influenza virus, and reovirus. A number of different reactors have been developed for cell culture, such as hollow fiber, packed-bed and fluidized-bed reactors, BelloCell (Cesco Bioengineering Co., Taiwan) [10], roller bottles, CellCube (Corning, USA), and Cell Factories (Nalge Nunc International, Denmark), and the use of Vero cells allows large-scale production of vaccines using microcarriers in a bioreactor.

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doi:10.1016/j.vaccine.2008.04.076

On one hand, culture media such as modified Eagle MEM, DMEM, and M199 are used for the cultivation of Vero cells adherent to microcarriers for the production of viral suspensions. Supplementation of these media with fetal bovine serum or human or bovine albumin is necessary for cell growth in bioreactors. However, the use of serum in cultures presents some disadvantages, such as the potential to induce hypersensitivity and the possible presence of contaminants (bacteria, fungi, mycoplasmas, bovine viruses, etc.) [11]. Furthermore, the recent threat to human health caused by the undefined agent of bovine spongiform encephalopathy (BSE) is likely to limit the continued use of bovine serum in culture processes applied for the synthesis of health-care products such as viral vaccines. Therefore, some culture media formulated without any human- or animal-derived components, designed to support the serum-free growth of several cell lines, have become widely utilized in virus production and biotechnology. Reovirus and rabies have been produced in Vero cells using serum-free medium [12,13], and JEV production was also attempted in 100 ml spinner flasks [14].

In this study, we produced a Vero cell-derived JE vaccine using medium with no components of human or animal origin in a 50-l fermenter, and compared it with a mouse brain-derived JE vaccine with respect to its physicochemical and immunological properties. In addition, the more stable Vero cell-derived vaccine condition was investigated by comparing the neutralization titers of vaccines with or without stabilizers during the inactivation and storage periods, stored at 4 °C or 28 °C for 1 year.

2. Materials and methods

2.1. Cell banks

Vero cells (CCL-81), a continuous African Green Monkey kidney cell line, were purchased from ATCC at passage number 121. For cell culture, VP-SFM (Invitrogen, USA) supplemented with 4 mM L-glutamine was used as serum-free, non-animal-derived nutrient medium. The cells were passaged directly into VP-SFM from serum-containing medium without any adaptation protocol. The cells were expanded and collected at passage number 126, and constituted the master cell bank (MCB). The MCB was dispensed into each vial (Nunc CryoLine System-CryoTubes, Nalgene Nunc Thermo Scientific, USA) using celltrol-4 (Finggal Link., Ltd., Japan), and stored in the vapor phase in a liquid-nitrogen container.

One vial of the MCB was thawed and expanded and constituted the working cell bank (WCB) at passage number 131, using the same method as for MCB. The MCB and WCB were tested according to WHO requirements for cell lines [8]: the absence of fungi, bacteria, mycoplasmas [15], and other adventitious agents, determination of the karyotype, and lack of tumorigenicity [16]. These biosafety tests of cell banks were carried out at CHARLES RIVER LABORATORIES, Inc. (USA).

The cells were used for experiments at passage number 132–140.

2.2. Virus banks

The Beijing-1 strain of JEV used for the production of the current mouse brain-derived JE vaccine was adapted to Vero cells using VP-SFM. The master virus bank (MVB) and working virus bank (WVB) were prepared at passage levels two and four, respectively. The MVB and WVB were dispensed into each vial (Micro tubes, Assist Co., Ltd., Japan), and stored at −80 °C. These banks were subjected to control tests such as viral identity with direct DNA sequencing analysis of cDNA covering the preM-E region, titration in Vero cells, and the absence of mycoplasmas, fungi, bacteria, and other adventitious agents, performed according to the Minimum Requirements for Biological Products for live vaccines in Japan [17] and FDA's draft guidance [18].

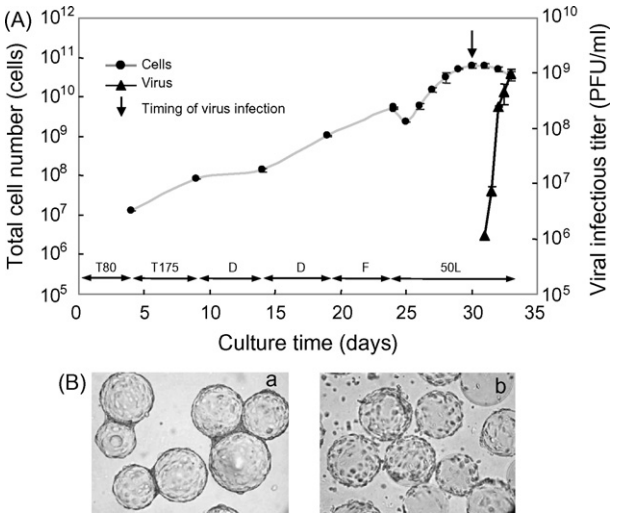

Fig. 1. (A) Profiles of Vero cell growth using VP-SFM from an 80-cm^2 flask to a 50-l fermenter, and JEV replication in a 50-l fermenter. (B) Photomicrographs of Vero cells on microcarriers at 6 days after the start of cell culture in a 50-l fermenter when JEV was inoculated (a) and at 3 days after commencing virus culture when virus production was harvested (b). (●) Total cell number, (▲) viral infectious titer, (T80) cell culture using an 80-cm^2 flask, (T175) cell culture using a 175-cm^2 flask, (D) cell culture using a double tray of CellSTACK and (F) cell culture using a Cell Factory, and (50 l) cell and virus culture using a 50-l fermenter.

2.3. Large-scale fermentation of Vero cells and production of JEV

Vero cells were grown on microcarriers in a 50-l fermenter (ABLE Co., Japan) using VP-SFM supplemented with 4 mM L-glutamine and TrypLE SELECT (Invitrogen) as a non-animal alternative for porcine trypsin, with 3 g/l Cytodex-1 (GE Healthcare Bio-Sciences, USA), prepared according to the manufacturer's instructions. One vial of the WCB was thawed and cultured in a culture flask (80 cm^2) using VP-SFM. The cells were harvested at full confluence by TripLE SELECT treatment, resuspended in VP-SFM, centrifuged at 1000 × *g*, resuspended in fresh medium, and inoculated in a larger culture flask (175 cm^2 or double tray of CellSTACK, Corning). When the cells were expanded to four Cell Factories (Nalgene Nunc Thermo Scientific, USA), the collected cells (5×10^9) were seeded into a 50-l fermenter containing 3 g/l of microcarriers and VP-SFM (Fig. 1). Cells were allowed to attach onto the surface of the microcarriers for 6.5 h with intermittent agitation at 18 rpm for 3 min every 30 min, and cell culture was commenced with 25 l of VP-SFM at 7 rpm agitation to maintain a high cell density and facilitate cell attachment to the microcarriers for 2 days. Two days after cell seeding, 25 l of VP-SFM was added and agitation was increased to 10 rpm. During culture, pH and residual glucose and glutamine concentrations were monitored twice a day. Glucose, lactate, glutamine, and glutamate in the culture supernatant were measured offline using a Bio Flow VER 5.0 (Oji Scientific Ins., Japan). When the glucose or glutamine concentration was low, half of the culture medium was exchanged with fresh VP-SFM (Fig. 2). pH was controlled at 7.2 through the addition of 7.5% $NaHCO_3$ or CO_2 gas to the medium.

For JEV production, the WVB was inoculated at a multiplicity of infection (MOI) of 0.01 when the cell number exceeded 1×10^6 cells/ml, usually 6 days after seeding. Microcarriers were allowed to settle by stopping the rotation, about 45 l of culture medium was removed, and the microcarriers were washed twice