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# Japanese encephalitis virus production in Vero cells with serum-free medium using a novel oscillating bioreactor

Hiroko Toriniwa, Tomoyoshi Komiya*

*Department of Research and Development, Research Center for Biologicals, The Kitasato Institute, 6-111 Arai, Kitamoto, Saitama 364-0026, Japan*

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**Abstract**

A novel oscillating bioreactor, BelloCell, was successfully applied for the cultivation of Vero cells using serum-free medium, and the production of Japanese encephalitis virus. The BelloCell requires no air sparging, pumping, or agitation, and thus provides a low shear environment. Owing to its simple design, BelloCell is extremely easy to handle and operate. Using this BelloCell (500 ml culture), Vero cells reached a maximum number of $2.8 \times 10^9$ cells and the Japanese encephalitis virus yield reached $6.91 \times 10^{11}$ PFU, versus $9.0 \times 10^8$ cells and $2.98 \times 10^{11}$ PFU using a spinner flask (500 ml) with microcarriers. The cell yield and virus production using BelloCell were markedly higher than with microcarrier culture. The neutralizing capacity of the Japanese encephalitis virus produced using BelloCell was equal to that using a microcarrier system. Therefore, these benefits should enable BelloCell to be adopted as a simple system for high population density cell culture and virus production.


*Keywords:* Japanese encephalitis virus; Vero cell; Serum-free medium; BelloCell

## 1. Introduction

Japanese encephalitis (JE) is a severe disease that is widespread throughout Asia, and is spreading beyond its traditional boundaries. Among 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.

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 used in the 1930s, and has been effective in reducing the incidence of JE. 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 developed. Vero cells derived from the African green monkey have been approved for viral vaccine production under specified regulatory guidelines [3,4], 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. The use of these cells also allows large-scale production of vaccines using microcarriers and culture in a bioreactor. The maximum cell population density achieved in these reactors, however, was limited at $1.8 \times 10^6$ cells/ml, because of limited oxygen transfer, toxic by-product accumulation, and the bead-to-bead collision effect under high shear agitation [5]. To overcome these problems, a number of different reactors have been developed, such as hollow-fiber and packed-bed bioreactors. Hollow-fiber reactors

*Abbreviations:* JE, Japanese encephalitis; JEV, Japanese encephalitis virus; UH, upper holding time; BH, bottom holding time; MOI, multiplicity of infection; dpi, days post-infection.

* Corresponding author. Tel.: +81 48 593 3953; fax: +81 48 593 3854.
*E-mail address:* komiya-t@kitasato.or.jp (T. Komiya).

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generate a low shear stress but require an exterior oxygenation system and a high circulation rate to provide adequate oxygen [6]. Packed-bed and fluidized-bed reactors are designed to maximize the cell population density, but sophisticated skills are required for their operation and they are expensive [7]. Other systems, such as roller bottles, CellCube (Corning, NY, USA) and Cell Factories (Nalge Nunc International, Roskilde, Denmark), are also available for immobilized cell culture; however, the cells are cultured on stacked surfaces and oxygen transfer might pose a potential problem.

Recently, a novel disposable bioreactor, BelloCell, composed of a packed-bed in the upper chamber and "bellows" in the lower chamber, was developed for animal cell culture (Cesco Bioengineering Co., Hsinchu, Taiwan). The oscillating compression and relaxation of the bellows enables the immobilized cells to be sequentially submerged into medium for nutrient supply and exposed to the ambient air for gas exchange. Chen et al. [8] and Wang et al. [9] have successfully applied this device to cultivate mammalian cells and produce HDV-like particles or a fusion protein, respectively.

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 the bioreactor. However, the use of serum in the cultures presents some disadvantages, such as the potential to induce hypersensitivity and the possible presence of contaminants (bacteria, fungi, mycoplasma, bovine viruses, etc.) [10]. 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 used for the synthesis of healthcare products such as viral vaccines. Therefore, some culture media formulated without any human- or animal-derived components, designed to support serum-free growth of several cell lines, have become widely utilized in virus production and biotechnology.

In this study, we achieved high population density cell culture and JEV production using BelloCell with serum-free medium. In addition, the performance of this system for Vero cell culture and JEV production was compared with that of a conventional spinner flask bioreactor with microcarriers, and we confirmed the neutralizing capacity of inactivated JEV produced using BelloCell.

## 2. Materials and methods

### 2.1. Cells, virus, and medium

Vero cells (CCL-81), a continuous African Green Monkey kidney cell line, were purchased from ATCC at passage number 121. The Beijing-1 strain of JEV was adapted for use in Vero cells. For cell culture and virus production, VP-SFM (Invitrogen, California, USA) supplemented with 4 mM L-glutamine was used as serum-free, non-animal-derived nutrient medium. Vero cells were grown through several passages using VP-SFM to adapt the medium before use, and the cells were used for experiments at passage number 130 to 140.

### 2.2. Carrier for cell immobilization

The carrier used for cell immobilization, BioNOCII (Cesco Bioengineering Co.), was non-woven fabric strips (width ~5 mm, length ~10 mm) made of 100% polyethyleneterephthalate (PET) with a specific surface area of about 2400 $cm^2/g$. The fabric was then specially surface-treated to make it hydrophilic and biocompatible. Each BelloCell, pre-packed with ~865 (bed volume ~100 $cm^3$) BioNOCII carrier chips and pre-sterilized by γ-irradiation, was purchased from Cesco Bioengineering Co.

### 2.3. BelloCell configuration and operation

The working liquid volume of the BelloCell bioreactor used in this study was 500 ml. The BelloCell consists of an upper chamber holding a carrier bed and a compressive lower chamber (bellows) containing medium, and its regulation is performed by the BelloStage, a control console, as shown in Fig. 1.

For cell inoculation, $1.5 \times 10^8$ cells were centrifuged at 1000*g* and resuspended in 50 ml fresh medium as the inoculum. After 450 ml medium was added into the bellows, 50 ml cell inoculum was applied to the carrier drop by drop, and the BelloStage maintained an up/down speed of 2.0 mm/s and an upper holding time (UH) of 20 s for adhesion of the cells with the BioNOCII carrier. After >95% of cells were attached to the matrix in about 6 h, the linear moving rate was set at an up/down speed of 1.0 mm/s with a bottom holding time (BH) of 1 min for cell growth. During culture, pH and residual glucose and glutamine concentrations were monitored twice a day. When glucose or glutamine concentration was low, the culture was replenished with fresh VP-SFM (Fig. 2). pH was controlled at 7.2 by adjusting $\%CO_2$ in the incubator and/or addition of $NaHCO_3$ in the medium.

### 2.4. Virus production using BelloCell

When the cell population density exceeded $1 \times 10^{10}$ cells, usually 7 days after seeding, JEV was inoculated at a multiplicity of infection (MOI) of 0.01. The spent medium was discarded and the immobilized cells were washed with 450 ml PBS for 10 min. After washing, PBS was discarded and virus inoculation was initiated by the addition of 300 ml virus solution for 1.5 h. The linear moving rate was set at 1 mm/s with UH of 15 min for virus adsorption to cells. At the end of the virus inoculation period, the virus solution was removed and 500 ml fresh VP-SFM was added. The linear moving rate was set at 1 mm/s with BH of 1 min.

During the virus production period, BelloCell was used for batch or semi-batch culture. In batch culture, virus was cultured for 3 or 4 days with no medium change, and the entire virus production was harvested when the viral titer was highest, usually 3 days post-infection (dpi). On the other hand, a semi-batch feeding strategy was adopted by changing 470 ml medium twice a day to

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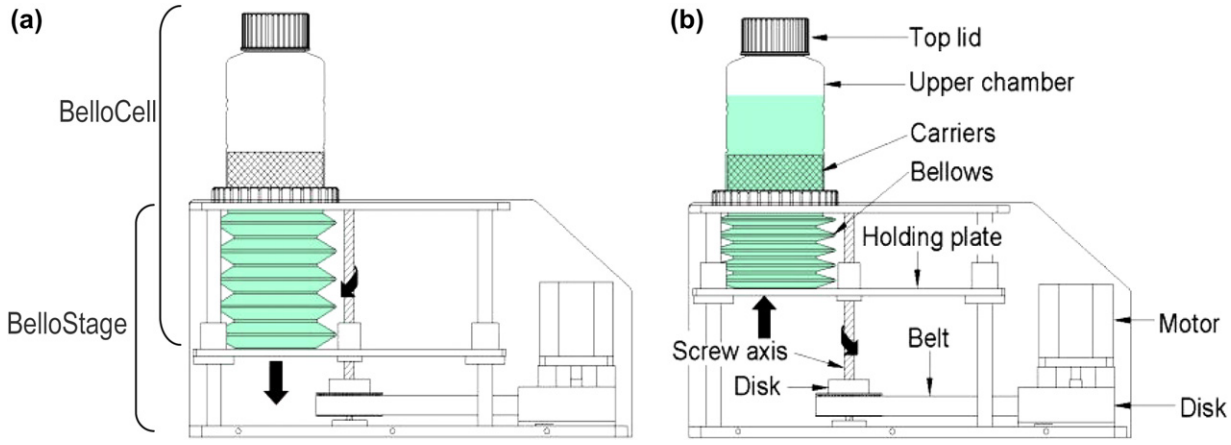


Fig. 1. Operating principles of BelloCell system. (a) The descending movement of the holding plate drops the medium onto the lower bellows, thus exposing the carrier to air for oxygen transfer. (b) After a time delay, the ascending movement of the holding plate lifts the bellows and raises the medium level to submerge the carrier, thus allowing nutrient transfer. The cyclic change in the rotating direction of the screw shaft moves the holding plate upward and downward.

maintain glucose and glutamine concentrations after 2 dpi. Then, the virus solution was kept at −80 °C until purification.

During virus production, culture conditions were maintained at pH 7.2 as described above and 37 °C in a $CO_2$ incubator.

### 2.5. Vero cell culture and virus production using spinner flask with microcarriers

Vero cells were grown on microcarriers in a 500-ml spinner flask (Techne, Staffordshire, UK) with 3 g/l Cytodex-1 (GE Healthcare Bio-Sciences, New Jersey, USA), prepared according to the manufacturer's instructions. Cells ($5 \times 10^7$) were allowed to attach onto the surface of Cytodex-1 for 4 h with intermittent agitation at 35 rpm for 3 min every 30 min, and cell culture was commenced with 250 ml VP-SFM at 35 rpm agitation to maintain a high cell population density and facilitate cell attachment to microcarriers. One day after cell seeding, medium was added to 500 ml. Four, 6 and 7 days after seeding, 250 ml culture medium was exchanged with fresh VP-SFM to maintain the nutrient levels (Fig. 3).

For JEV production, the virus solution was inoculated at MOI of 0.01 when the cell population density exceeded $1 \times 10^6$ cells/ml. Microcarriers were allowed to settle by stopping the rotation, about 450 ml culture medium was removed, and the microcarriers were washed with 100 ml PBS and fresh VP-SFM. After discarding the VP-SFM, the culture was infected by adding 100 ml JEV solution, and the flask was agitated intermittently at 35 rpm for 3 min every 15 min for 1.5 h. After discarding the excess virus solution, fresh VP-SFM was added to the flask and the cells were incubated at 37 °C while maintaining pH at 7.2 in a $CO_2$ incubator. During virus production, the viral culture was used for batch or semi-batch culture as with the BelloCell culture.

### 2.6. Analytical method

Glucose, lactate, glutamine, and glutamate in the culture supernatant were measured offline using a Bio Flow VER5.0 (Oji Scientific Ins., Hyogo, Japan).

To measure cell density, the crystal violet dye (CVD) nucleus staining method was used as described by Ho et al.

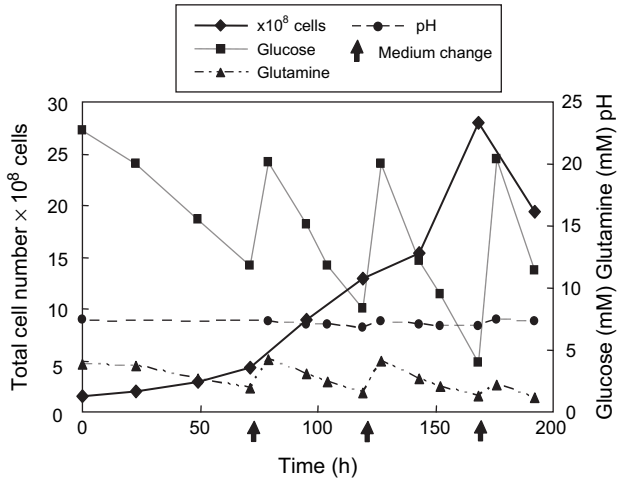


Fig. 2. Cell growth, glucose and glutamine concentrations, and pH using BelloCell bioreactor. Arrows show changes of medium.

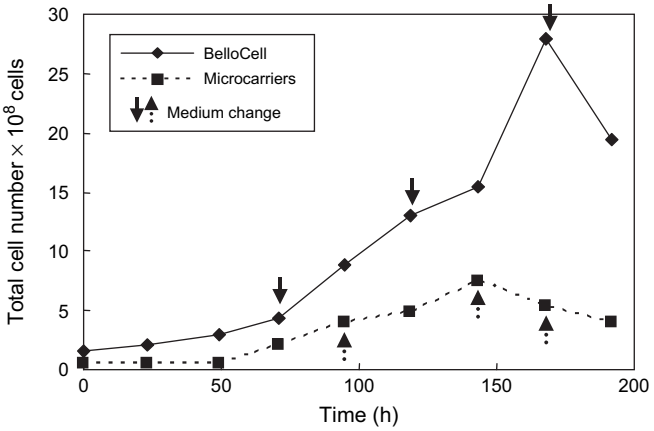


Fig. 3. Profiles of Vero cell growth using BelloCell and spinner flask with microcarriers. Upward arrow show a medium change of BelloCell culture, and downward arrow show a medium change of microcarrier culture.

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[11]. Two disks each was sampled from four different locations in the bottle, placed in a 1.5-ml tube, and 1.0 ml CDV reagent was added.

The titer of JEV was determined as plaque forming units (PFU) in Vero cells, as described by Toriniwa and Komiya [12].

Experiments on cell culture or virus production were each performed five times, and the cell population density or virus titer was expressed as mean ± standard deviation.

### 2.7. Virus purification and inactivation

Each pooled culture medium from viruses harvested using BelloCell or microcarrier culture was filtered using a 0.22-μm filter to remove cells and cell debris. The filtrate was concentrated to about 1/10 of the original volume with a membrane with a size exclusion limit of 100 kDa (Biomax-100, Millipore Co., MA, USA). Dilution with PBS and concentration were repeated several times to remove low molecular weight impurities. After filtration using a 0.22-μm filter, 40% ethanol was added to a final concentration of 15%, and incubation was performed at 4 °C for 8–16 h. The virus solution was centrifuged at 32,950*g* at 4 °C for 70 min, and the precipitate was suspended in PBS. After filtration using a 0.22-μm filter, formalin was added to a final concentration of 0.05% and the virus solution was inactivated for 3 months at 4 °C.

After inactivation, the virus solution was filtered through a 0.22-μm filter and subjected to 20% and 60% sucrose gradient ultracentrifugation at 100,000*g*, 4 °C, for 5 h. The fractions containing virus antigen were pooled, and dialyzed against PBS, filtered through a 0.22-μm filter, and additives were added to the dialysate to formulate experimental vaccines.

### 2.8. Immunogenicity test

An immunogenicity test was performed with mouse immunization and the virus neutralization titer of the antiserum, according to the Japanese minimum requirements for the current JE vaccine [13] using 4–5-week-old female ddY mice. Following purification of JEV produced using the BelloCell or spinner flask method, these experimental vaccines and reference vaccine (mouse brain-derived comparator vaccine, Lot No. 105 from National Institute of Infectious Diseases, Japan) were diluted to 3 μg/ml with PBS, and mice were inoculated twice with 0.5 ml of these diluted vaccines by the intraperitoneal route at 1-week intervals. One week after the second injection, the mice were bled and the sera were pooled and heat-inactivated at 56 °C for 30 min before use. The virus neutralization titer of the antiserum was assessed by 50% plaque reduction method as described by Toriniwa and Komiya [12].

## 3. Results

### 3.1. Cell growth in BelloCell

To investigate the feasibility of high population density cell cultivation using BelloCell, Vero cells were placed in the bioreactor with a total medium volume of 500 ml. For cell inoculation, Vero cells suspended in 50 ml fresh medium as the inoculum were dropped directly onto carrier chips, and the BelloCell was operated by the BelloStage. The most effective inoculum density was determined to be $1.5 \times 10^8$ cells (i.e., $3 \times 10^5$ cells/ml), because this led to the highest cell number in the shortest period (data not shown). Based on desirable glucose and glutamine concentrations, the timing of exchange of medium was determined as 3, 5, and 7 days after the start of culture (Fig. 2). The average maximum cell number was $28.0 \pm 0.98 \times 10^8$ after 7 days (Fig. 2, Table 1). On the other hand, when Vero cells were cultured in a spinner flask containing 3 g/l microcarriers, the average maximum cell number was $9.0 \pm 1.27 \times 10^8$ cells after 6 days (Fig. 3). Thus, the BelloCell provided a higher cell population density system for Vero cell culture.

### 3.2. Virus production using BelloCell

Since a high cell culture rate could be attained with the BelloCell, the capability for JEV production was tested. The cells were grown similarly to as described above to $1.0 \times 10^{10}$ cells for 7 days, and infected with JEV at MOI of 0.01. Virus production was best at MOI of 0.01 (data not shown). The viral titer remained low on the first and second dpi and then increased markedly from 2 to 3 dpi. As the virus recovery system, two methods were examined: batch culture and semi-batch culture. Batch culture recovers the medium all at once when the viral titer is highest, and semi-batch recovery involves a feeding strategy for viral recovery and 470 ml medium is changed each time from 2 to 4 dpi. The average JEV titer was $2.73 \pm 1.03 \times 10^{11}$ PFU at 3 dpi with batch culture, whereas with semi-batch culture, the average total viral yield was $6.91 \pm 0.96 \times 10^{11}$ PFU for 4 days (Table 1).

For comparison, virus production was also performed in a spinner flask with 1.5 g microcarriers (500 ml working volume). In batch culture, the average maximum viral titer was only $2.98 \pm 1.04 \times 10^{11}$ PFU at 3 dpi, whereas the average total virus production was $1.23 \pm 1.11 \times 10^{11}$ PFU in semi-batch

Table 1
Comparison of Vero cell growth and JEV production using BelloCell and microcarriers

| | BelloCell 500 | Microcarrier 500 |
|---|---|---|
| Total surface area ($cm^2$) | 15,600 | 9,000 |
| Medium (ml) | 500 | 500 |
| Max. cell population density ($10^6$ cells/ml)[a] | 5.60 ± 0.20 | 1.80 ± 0.25 |
| Total cell number ($10^8$ cells)[a] | 28.0 ± 0.98 | 9.00 ± 1.27 |
| Multiplicity of infection (MOI) | 0.01 | 0.01 |
| Total virus production/batch ($10^{11}$ PFU)[b] | 2.73 ± 1.03 | 2.98 ± 1.04 |
| Total virus production/semi-batch ($10^{11}$ PFU)[b] | 6.91 ± 0.96 | 1.23 ± 1.11 |
| Neutralization titer (3 μg/ml)[c] | 2.958 | 2.469 |
| Neutralization titer of reference (3 μg/ml)[c] | 2.448 | 2.448 |

[a] The cell population density or number was expressed as mean ± SD.
[b] The virus titer was expressed as mean ± SD.
[c] The neutralization titer was expressed as $\log_{10}$.

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culture (Table 1). Thus, for microcarrier culture, viral culture was more productive with batch culture than with semi-batch culture. Thus, the viral concentration produced in the BelloCell was higher than that produced in the spinner flask with microcarriers.

### 3.3. Immunogenicity

To confirm the immunogenicity of JEV produced using BelloCell, inactivated purified JE virus was injected into mice intraperitoneally. The virus neutralization titers of sera obtained by immunization with JE virus produced using the BelloCell or a spinner flask exceeded the titer developed with mouse brain-derived JEV (Table 1). These data indicate that JEV produced using BelloCell exhibited immunogenicity consistent with that produced by a commercial vaccine.

## 4. Discussion

Considering that the proliferation of cells supporting viral replication is important to increase the efficiency of virus production, we evaluated large-scale methods of viral culture. For large-scale culture, various bioreactors such as the hollow-fiber bioreactor, Celligen Plus Bioreactor (New Brunswick Scientific, Edison, NJ), and Wave Bioreactor (Wave Biotech, Somerset, NJ) are available, but all of them involve complicated operation, and provide low population densities of cells. Roller Bottles, CellCube and Cell Factory systems are available for the culture of anchorage-dependent cells, but these culture methods also limit cell proliferation. Microcarriers are often used for cell culture; however, Cesco Bioengineering Co. (Hsinchu, Taiwan) has recently developed the BelloCell bioreactor, a simpler high population density cell culture system. We compared cell culture in this bioreactor with conventional microcarrier culture.

To determine the conditions of culture in the BelloCell, we measured glucose and glutamine levels during culture of Vero cells. Starting with $1.5 \times 10^8$ cells, we found that a change of medium every other day was necessary from 3 days after the start of culture to maintain the concentration of each nutrient at or above 1 mmol/l. Under these conditions, the total number of cells reached $2.8 \times 10^9$ cells with $5.6 \times 10^6$ cells/ml at 168 h after the start of culture. When cells were cultured in the BelloCell at an initial $5 \times 10^7$ cells/ml, as in microcarrier culture, the total number of cells was lower, $1.1 \times 10^9$ cells, than that in culture with an initial cell number of $1.5 \times 10^8$ cells. Therefore, it appeared to be more effective to start culture in the BelloCell with $1.5 \times 10^8$ cells (data not shown). On the other hand, when microcarrier culture in a 500-ml spinner flask was started with $5 \times 10^7$ cells, the total number of cells reached $9.0 \times 10^8$ cells with $1.8 \times 10^6$ cells/ml after 6 days of culture. When cells were cultured with an initial cell number of $1.5 \times 10^8$ cells as in BelloCell culture, the maximum cell number was the same, and it only shorted the culture period (data not shown). Previous studies have shown that microcarrier culture of Vero cells in serum-containing medium yielded $1-1.56 \times 10^6$ cells/ml [14,15]. Cell culture with microcarriers at 5 g/l did not increase the yield of Vero cells above that achieved with 3 g/l (data not shown). Wu et al. [16] reported that microcarrier culture with 10 g/l microcarriers gave $1.9 \times 10^6$ cells/ml, which was considerably lower than that with the present BelloCell culture. Other studies achieved $7.6 \times 10^6$ and $9.6 \times 10^6$ cells/ml; however, the viral culture resulted in a very low viral yield, presumably because of the inability to maintain their nutritional state [16,17]. Using serum-free medium, $1.6 \times 10^6$ cells/ml was achieved [16], which was comparable to that obtained in the present study. In comparison with BelloCell culture and microcarrier culture, cell proliferation rates of 18.7 and 18, respectively, were effectively the same. Moreover, cell number per surface area, $1.79 \times 10^6$ cells/cm$^2$ in BelloCell culture and $1.00 \times 10^6$ cells/ml in microcarrier culture, were almost the same. However, BelloCell culture using the same volume (500 ml) of medium as that in microcarrier culture yielded about 3 times more cells. Thus, compared with these reported microcarrier cultures, the BelloCell culture system enables much higher cell population densities to be achieved. To supply the oxygen needed in microcarrier culture, the flask requires vigorous agitation, which imposes physical stress on the cells [5]. In contrast, the BelloCell culture system effectively supplies oxygen and imposes little stress, allowing the maintenance of a higher cell count [18]. Because Vero cells are adherent, it is important to increase the surface area for attachment of cells. The BelloCell system provides a large surface area and high population density cell culture.

In this study, we compared two methods of supplying culture medium for the production of JEV: batch culture involves the cultivation of cells without a change of medium after virus inoculation, whereas semi-batch culture is carried out by changing the medium every 8 and 15 h from 2 days after the start of culture of viruses, when viruses are rapidly replicating. The amount of virus with batch and semi-batch culture in the BelloCell was $2.73 \times 10^{11}$ and $6.91 \times 10^{11}$ PFU, respectively. Similarly, the amount of virus with batch and semi-batch culture on microcarriers was $2.98 \times 10^{11}$ and $1.23 \times 10^{11}$ PFU, respectively. Thus, in contrast to microcarrier culture, semi-batch culture gave a higher viral yield than batch culture in the BelloCell. It has been reported that when Vero cells were cultured on Cytodex-1 microcarriers for JEV production, $0.1-1 \times 10^9$ PFU/ml ($0.5-5 \times 10^{11}$ PFU) of virus was obtained [14,15,19]. These observations indicate that BelloCell culture could achieve higher total virus production. By allowing high population density culture along with a highly efficient oxygen supply, BelloCell culture is favorable for virus production. In addition, the low physical stress in the system may also help to protect virus released into the supernatant [18]. In addition, cell culture is maintained at a higher level, and virus production is maintained at a higher rate for longer in semi-batch culture using the BelloCell than in batch culture using the BelloCell or microcarrier culture. Therefore, we speculate that the reason for the high viral yield while cells are not in contact with viruses and are well supplied with nutrients to proliferate is that infected cells continue to produce viruses. While semi-batch culture requires more medium than

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batch culture, the total cost for JEV production in semi-batch culture was lower than that in batch culture.

Furthermore, JEV produced in the BelloCell was concentrated, purified, inactivated, and injected into mice to test the ability to produce neutralizing antibodies as a vaccine. When mice were immunized with the same amount of protein of this JEV as that in the current mouse brain-derived and microcarrier culture-derived vaccines, we found that it led to a higher titer of antibody than with the mouse brain-derived vaccine and approximately the same titer of antibody as with microcarrier culture-derived JEV. These results confirm that BelloCell culture is a new JEV culture method that can be expected to replace microcarrier culture, and suggest that it can be utilized for the production of vaccines.

For the past 50 years or so in Japan, highly purified, safe, increasingly effective, mouse brain-derived vaccines have been used; however, the possibility cannot be excluded that, during manufacture, these vaccines are contaminated with mouse brain-derived impurities, the components of which may cause ADEM. Thus, there is a need to develop a JE vaccine by tissue culture, which involves a low risk of contamination with impurities. Cell culture media such as MEM, DMEM, and M199 are generally used, and need to be supplemented with FBS or BSA to promote greater cell proliferation. However, because of the recent BSE issue, serum-free media containing no animal-derived components have been utilized to minimize the risk of contamination. By a new culture technique using the BelloCell culture system and serum-free medium, VP-SFM, we successfully produced a larger amount of JEV than has been reported with other culture systems using Vero cells currently approved for the production of poliovirus and rabies vaccines. Since the BelloCell system needs no air sparging, pumping, or agitation, it provides a low shear environment, and it is simple to operate and easy to handle because of its simple design. The use of this BelloCell bioreactor will facilitate large-scale production of viruses. Cesco Bioengineering Co. has developed a 25-l TideCell bioreactor that uses similar principles to the BelloCell. Larger-scale culture of cells or virus for vaccine production can be expected using the TideCell.

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## References

[1] Burke DS, Monath TP. Fields Virology. 4th ed. Lippincott Philadelphia; 2001. p. 991–1055.
[2] Tsai TF. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand, 13–15 October 1998. Vaccine 2000;18:1–25.
[3] World Health Organization. Requirements for continuous cell lines used for biological substances. WHO Tech Rep Ser 1987;745:99–115.
[4] World Health Organization. Acceptability of cell substrates for production of biologicals. WHO Tech Rep Ser 1987;747:5–24.
[5] Mendonca RZ, Pereira CA. Cell metabolism and medium perfusion in Vero cell cultures on microcarriers in a bioreactor. Bioprocess Eng 1998; 18:213–8.
[6] Alauotila S, Marjamaki A, Matikainen MT, Jalkanen M. Use of a hollow-fiber bioreactor for large-scale production of alpha 2-adrenoceptors in mammalian cells. J Biotechnol 1994;37:179–84.
[7] Hu Y-C, Kaufman J, Cho MW, Golding H, Shiloach J. Production of HIV-1 gp120 in packed-bed bioreactor using the vaccinia virus/T7 expression system. Biotechnol Prog 2000;16:744–50.
[8] Chen YH, Wu JC, Wang KC, Chiang YW, Lai CW, Chung YC, et al. Baculovirus-mediated production of HDV-like particles in BHK cells using a novel oscillating bioreactor. J Biotechnol 2005;118:135–47.
[9] Wang IK, Hsieh SY, Chang KM, Wang YC, Chu A, Shaw SY, et al. A novel control scheme for inducing angiostatin-human IgG fusion protein production using recombinant CHO cells in an oscillating bioreactor. J Biotechnol 2006;121:418–28.
[10] Froud SJ. The development, benefits and disadvantages of serum-free media. Dev Biol Stand 1999;99:157–66.
[11] Ho L, Greene CL, Schimidt AW, Huang LH. Cultivation of HEK 293 cell line and production of a member of the superfamily of G-protein coupled receptors for drug discovery applications using a highly efficient novel bioreactor. Cytotechnology 2004;45:117–23.
[12] Toriniwa H, Komiya T. Rapid detection and quantification of Japanese encephalitis virus by real-time reverse transcription loop-mediated isothermal amplification. Microbiol Immunol 2006;50:379–87.
[13] Japanese Encephalitis vaccine. In: Association of biologicals manufacturers of Japan (eds). Minimum requirements for biological products; 1993. p. 9–101.
[14] Sugawara K, Nishiyama K, Ishikawa Y, Abe M, Sonoda K, Komatsu K, et al. Development of Vero cell-derived inactivated Japanese encephalitis vaccine. Biologicals 2002;30:303–14.
[15] Appaiahgari MB, Vrati S. Immunogenicity and protective efficacy in mice of a formaldehyde-inactivated Indian strain of Japanese encephalitis virus grown in Vero cells. Vaccine 2004;22:3669–75.
[16] Wu SC, Liu CC, Lian WC. Optimization of microcarrier cell culture process for the inactivated enterovirus type 71 vaccine development. Vaccine 2004;22:3858–64.
[17] Berry JM, Barnabe N, Coombs KM, Butler M. Production of reovirus type-1 and type-3 from Vero cells grown on solid and macroporous microcarriers. Biotechnol Bioeng 1999;62:12–9.
[18] Hu YC, Lu JT, Chung YC. High-density cultivation of insect cells and production of recombinant baculovirus using a novel oscillating bioreactor. Cytotechnol 2003;00:1–9.
[19] Wu SC, Huang GYL. Stationary and microcarrier cell culture processes for propagating Japanese encephalitis virus. Biotechnol Prog 2002;18: 124–8.

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