Vaccine 28 (2010) 6951–6957

Contents lists available at ScienceDirect

Vaccine

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

Enterovirus 71 virus-like particle vaccine: Improved production conditions for enhanced yield

Cheng-Yu Chung[a], Chi-Yuan Chen[a], Shih-Yeh Lin[a], Yao-Chi Chung[a], Hsin-Yi Chiu[a], Wei-Kuang Chi[b], Yu-Li Lin[c], Bor-Luen Chiang[d], Wei-Jheng Chen[a], Yu-Chen Hu[a,*]

[a] Department of Chemical Engineering, National Tsing Hua University, 101, Sec. 2, Kuang Fu Rd., Hsinchu 300, Taiwan
[b] Development Center for Biotechnology, Taipei 221, Taiwan
[c] Department of Medical Research, National Taiwan University Hospital, Taipei 100, Taiwan
[d] Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei 100, Taiwan

ARTICLE INFO

Article history:
Received 5 May 2010
Received in revised form 9 August 2010
Accepted 9 August 2010
Available online 24 August 2010

Keywords:
3CD protease
Baculovirus
Bioprocess
Enterovirus 71
Virus-like particles

ABSTRACT

To develop the enterovirus 71 (EV71) vaccine, we previously constructed a recombinant baculovirus (Bac-P1-3CD) co-expressing EV71 P1 (under *polyhedrin* promoter) and 3CD (under *p10* promoter) proteins, which caused P1 cleavage by 3CD protease and self-assembly of virus-like particles (VLPs) in Sf-9 cells. Assuming that reducing the 3CD expression can alleviate the competition with P1 expression and elevate the VLPs yield, hereby we constructed Bac-P1-C3CD and Bac-P1-I3CD expressing 3CD under weaker CMV and IE-1 promoters, respectively. Western blot and ELISA analyses revealed that Bac-P1-C3CD and Bac-P1-I3CD led to the VLPs release into the supernatant and enhanced the extracellular VLPs yield in Sf-9 cells, but gave poor VLPs production in High Five™ (Hi-5) cells. By optimizing the process parameters including host cells, cell density, culture mode and dissolved oxygen (DO), the best extracellular VLPs yield was achieved by infecting Sf-9 cells (4×10^6 cells/mL) cultured in the bioreactor (DO = 30%) with Bac-P1-C3CD, which approached ≈64.3 mg/L and represented a ≈43-fold increase over the yield (1.5 mg/L) attained using the old process (Bac-P1-3CD infection of Sf-9 cells in the spinner flasks). The resultant VLPs not only resembled the VLPs produced from Bac-P1-3CD infection in density, size and shape, but also induced potent antibody responses in mouse models. The antibodies neutralized EV71 strains of homologous and heterologous genogroups, implicating the potential of the VLPs to confer cross-protection for the prevention of future epidemics. Altogether, Bac-P1-C3CD and the bioprocess render mass production more economical, obviate the need for cell lysis and hold promise for future industrial vaccine production.

1. Introduction

Enterovirus 71 (EV71) is a non-enveloped virus with a single-stranded RNA genome consisting of P1, P2 and P3 regions. P1 region encodes the P1 precursor while P2 and P3 regions encode non-structural proteins such as 3CD protease which can cleave P1 into VP1, VP3 and VP0. These three proteins spontaneously assemble into icosahedral procapsids and pack the RNA genome into the provirions [1]. VP1 is the major antigen and is highly variable among enteroviruses. Based on the sequences of VP1 gene, EV71 is divided into genogroups A, B and C [2] and subgenogroups within genogroups B and C (B1–B5 and C1–C5).

EV71 is the major etiological agent responsible for hand-foot-and-mouth disease (HFMD) in young children and infants, and children under 5 years of age are particularly susceptible to severe forms of EV71-associated neurological complications such as aseptic meningitis, brain stem encephalitis and even death [3]. Since the 1998 outbreak in Taiwan that caused 405 severe cases and 78 deaths [4], in recent years the Asia-Pacific region (e.g. Taiwan, China, Malaysia, Japan and Vietnam) has experienced more frequent EV71-associated HFMD epidemics with high incidence of neurotropic complications and fatality rates [3]. In 2008, the HFMD outbreak in Taiwan resulted in 373 severe cases and 14 deaths, all due to EV71 infection. The 2008 outbreak in China also led to 489,097 reported cases that included 1125 severe cases, and claimed 126 lives. From January to July 2009, the large-scale HFMD outbreak in China gave rise to 787,070 reported cases that included 10,509 severe cases and caused 255 deaths. The increasing frequency of EV71 epidemics and high fatality rates underscore the urgent need to develop the vaccines.

Currently, inactivated EV71 virus is the preferred vaccine type and is being actively developed. The virus can be grown in cell cul-

* Corresponding author. Tel.: +886 3 571 8245; fax: +886 3 571 5408.
E-mail address: yuchen@che.nthu.edu.tw (Y.-C. Hu).

0264-410X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2010.08.052

ture and inactivated by formalin [5] or heat [6]. Alternatively, the virus can be attenuated by serial passaging [7] or genetic modification [8]. However, these whole virus vaccines raise higher safety concerns. Given the potent immunogenicity, VP1 is another vaccine antigen of interest, which can plasmid-borne [6] or produced by *E. coli* [9], from transgenic tomato [10] or from the milk of transgenic mice [11]. These VP1-based DNA and subunit vaccines, however, are either not evaluated for vaccine efficacies or elicit poorer immune responses than the inactivated virus vaccines [12], probably because VP1 alone lacks certain conformation-dependent epitopes located at the junctions of structural proteins [13].

Virus-like particles (VLPs) are empty particles comprised of viral structural proteins. Thanks to the preservation of virus structure, VLPs can elicit broad and strong immune responses [14] and are promising vaccine candidates [15]. The majority of VLPs are produced using the baculovirus/insect cell expression system, which involves the infection of insect cells with a recombinant baculovirus that drives the foreign gene expression by the very late strong promoters *polyhedrin* (*polh*) or *p10* [16]. Traditionally, Sf-9 cell derived from *Spodoptera frugiperda* is the most widely utilized host cell, but BTI-TN-5B1-4 cell (trade name High Five™, Invitrogen) derived from *Trichoplusia ni* has gained popularity and employed for the production of commercial human papillomavirus VLP vaccine (Cervarix™, GlaxoSmithKline).

We have previously constructed recombinant baculoviruses Bac-P1 and Bac-3CD to carry EV71 P1 and 3CD genes, and co-infection of Sf-9 cells with these two viruses resulted in the self-assembly of EV71 VLPs within the cells [17]. In a follow-up study, we further constructed another baculovirus Bac-P1-3CD, which harbored both P1 and 3CD genes under the control of *polh* and *p10* promoters, respectively [18]. Bac-P1-3CD infection of Sf-9 cells led to the cleavage of P1 polyprotein by 3CD protease into VP0 (36 kDa), VP1 (39 kDa) and VP3 (26 kDa), and EV71 VLPs formation. After ultracentrifugation purification, the EV71 VLPs were indistinguishable from the authentic virus in size, appearance, composition and surface epitopes [18] and induced potent, long-lasting and cross-reactive humoral responses in immunized mice [19]. Importantly, the VLP immunization of mother mice conferred protection to neonatal mice against lethal viral challenge [19]. These data collectively implicate the potential of EV71 VLPs as a vaccine candidate. However, one bottleneck to the development of EV71 VLP vaccine was the relatively low total VLP yield [18]. To lift the roadblock to vaccine development, this study sought to improve the production yield by changing the baculovirus design and process parameters.

2. Materials and methods

2.1. Preparation of recombinant baculoviruses

The P1 and 3CD gene fragments were obtained by PCR from the infectious cDNA clone of EV71 YN3 strain (a branch strain of EV71 *neu* strain). The P1 gene was cloned into MCS (multiple cloning site) I of pFastBac DUAL vector (Invitrogen) under the *polh* promoter and the 3CD gene was cloned into MCS II under the *p10* promoter. The resultant pBac-P1-3CD plasmid was used to generate Bac-P1-3CD virus [18]. To construct pBac-P1-C3CD and pBac-P1-I3CD, the full-length cytomegalovirus immediately early (CMV) promoter and IE-1 promoter were PCR-amplified from pcDNA3.1 (−) vector (Invitrogen) and pIEX-1 vector (Merck), respectively, using primers encoding the *Bst*1107 I and *Sma* I enzyme sites. The *p10* promoter was deleted from pBac-P1-3CD by enzyme digestion, followed by the insertion of CMV or IE1 promoter sequences by *Bst*1107 I/*Sma* I treatment. The resultant plasmids were employed to prepare Bac-P1-C3CD and Bac-P1-I3CD viruses, respectively, using the Bac-To-Bac® system (Invitrogen). The recombinant viruses were propagated by infecting Sf-9 cells (FIRDI, Hsinchu, Taiwan) cultured in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco), harvested and titered by end-point dilution method [17].

2.2. EV71 virus preparation and purification

The EV71 virus strains used in this study included TW2272/98 (C2 genogroup, isolated in 1998) and 20080738 (B5 genogroup, isolated in 2008). Both virus strains were propagated in RD (rhabdomyosarcoma) cells cultured at 37 °C using Alpha Minimum Essential Medium (α-MEM, Invitrogen) containing 5% FBS. The virus harvested from the infected cells was precipitated with 14% polyethylene glycol 8000 (Sigma), and then purified by ultracentrifugation on a 25–40% discontinuous sucrose gradient (100,000 × *g* for 4 h). The band containing the virus was pelleted at 100,000 × *g* for 2 h and then resuspended in phosphate-buffered saline (PBS). The virus titers were determined based on the cytopathic effect (CPE) developing in infected RD cells [19] and are expressed as tissue culture infectious dose ($TCID_{50}$). The purified virus was heat-inactivated at 56 °C for 30 min and measured for the protein content using the Protein Assay kit (Bio-Rad).

2.3. VLP production and purification

The EV71 VLPs were produced by infecting Sf-9 or Hi-5 cells (Invitrogen, Cat. No. B855-02) cultured in SF-900 II medium with the recombinant baculovirus at a multiplicity of infection (MOI) 10. For small-scale production, the cells were cultured in 250 ml spinner flasks with 150 ml working volume. The VLPs-containing supernatant was harvested at different times by centrifugation (1000 × *g* for 30 min).

For VLPs production and subsequent purification, the cells were cultured in a 2 L stirred tank bioreactor (Biostat B®, B. Braun Biotech). The cells were inoculated at 1×10^6 cells/mL, grown to 4×10^6 cells/mL and then infected with Bac-P1-C3CD at MOI 10. The dissolved oxygen (DO) was controlled at 15%, 30% or 60% of air saturation with the built-in control system, by sparging a mixture of air and oxygen through bubble-free aeration system. At 4 days post-infection (dpi), the supernatant was harvested by centrifugation and loaded to the tangential flow ultrafiltration unit (Pellicon XL 50 cassette, molecular weight cut-off = 1000 KDa, Millipore) for concentration (to 1/10 of the original volume) and buffer exchange to TNE buffer (40 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4). The diafiltered sample was loaded to a 20–30% discontinuous CsCl gradient, which resulted in fractions with densities ranging from 1.28 g/cm^3 to 1.45 g/cm^3 after 24 h ultracentrifugation (100,000 × *g*, P40ST rotor, CP100MX, Hitachi). The fractions were collected from bottom to top (0.6 ml per fraction) and the sample in each fraction was pelleted (100,000 × *g* for 2 h), resuspended in PBS and analyzed by Western blot (see below). The purified VLPs were quantified for protein content using the Protein Assay kit (Bio-Rad) and examined by transmission electron microscopy (TEM) as described [18].

2.4. VLP analyses by Western blot and enzyme-linked immunosorbent assay (ELISA)

The VLPs were subjected to 12% SDS-PAGE and Western blot following the procedures described earlier [18], except that the primary antibody was a new mouse anti-VP1 polyclonal antibody (1:1500 dilution, provided by Prof. Bor-Luen Chiang). The secondary antibody was goat anti-mouse IgG conjugated with alkaline phosphatase (Kirkegaard and Perry Laboratories, KPL). The