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Large-Scale Production of Rotavirus VLP as Vaccine Candidate Using Baculovirus Expression Vector System (BEVS)

PARK, JIN YONG[1,2], HUN KIM[1,2], HI KU HWANG[1], SU JEEN LEE[1], HYUN SUNG KIM[1,3], BYUNG KI HUR[3], YEON WOO RYU[2], CHANG NAM AN[1], AND JONG SOO KIM[1]*

[1]*Vaccine Research Institute, GreenCross Vaccine Corp., 227-3 Gugal-Ri, Yongin-City 449-903, Korea*
[2]*Department of Molecular Science and Technology, Ajou University, Suwon 442-749, Korea*
[3]*Department of Biotechnology and Bioengineering, Inha University, Incheon 402-751, Korea*

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Abstract Rotavirus virus-like particle (VLP) composed of VP2, VP6, and VP7 was expressed in the Baculovirus Expression Vector System (BEVS). *Sf9* cell, a host of the baculovirus, was cultured from a 0.5-l spinner flask to the 50-l bioreactor system. *Sf9* cell was maintained at cell density between 3.0E+05 and 3.0E+06 cells/ml and grew up to 1.12E+07 cells/ml in the bioreactor. Growth kinetics was compared under different culture systems and showed similar growth kinetics with 20.1-25.2 h of doubling time. Early exponentially growing cell culture was infected with three recombinant baculoviruses expressing VP2, VP6, and VP7 protein at 1.0, 2.0, and 0.2 moi, respectively. The expression of rotavirus proteins was confirmed by Western blot analysis and its three-layered virus-like structure was observed under an electron microscope. Rotavirus VLP was semipurified and immunized in ICR mice intramuscularly. Rotavirus-specific serum antibody was detected from 2 weeks after the immunization and lasted at least 21 weeks of the post-immunization, indicating its possible use as a vaccine candidate.

Key words: Rotavirus, virus-like particle, baculovirus expression vector system, vaccine, *Sf9* insect cell

Rotavirus is one of the most important etiologic agents of diarrheal disease in infants and young children worldwide [2, 16]. Rotavirus infections occur as early as 2-3 months after birth with the highest frequency of gastroenteritis occurring between 6 and 18 months of age. More than 90% of all children have had at least one rotavirus infection by the age of three.

Rotavirus is a member of the Reoviridae family and composed of 6 structural proteins organized in three concentric shells surrounding 11 segmented double-stranded RNA. Only the two outer capsid proteins, VP4 and VP7, induce neutralizing antibodies [5]. The correlation of serum neutralizing antibodies to the protection, however, is still unclear. Rotaviruses are generally characterized based on VP7 serological relationships (G serotype). Currently, there are 14 known G serotypes, four of which (serotypes 1-4) are responsible for 95% of all human disease. Presently, serotype 1 is the most common serotype circulating in the world, followed by serotypes 3, 2, and 4 [14].

Baculovirus belong to a diverse group of the large double-stranded DNA viruses that infect many different species of insects as their natural hosts. Baculovirus strains are highly species-specific and are not known to propagate in any non-invertebrate host. The baculovirus genome is replicated and transcribed in the nuclei of infected host cells where the large baculovirus DNA (between 80-200 Kbp) is packaged into the rod-shaped nucleocapsid. Since the size of these nucleocapsids is flexible, recombinant baculovirus particles can accommodate large amounts of foreign DNA. AcNPV is the most extensively studied baculovirus strain [7]. Since the application of recombinant baculovirus for the production of recombinant protein in 1980s [18], BEVS has been used for vaccine research, including human papilomavirus VLP [10], porcine parvovirus VLP [13], recombinant therapeutics, and mammalian cell gene delivery vector [11]. Most rotavirus vaccine research is focused on live attenuated vaccine including neonatal strains or reassortant vaccines. Live oral vaccine is not efficacious for providing a full protection against mild disease [2], and association with intussusception has been reported [8]. New vaccine research including subunit vaccine, inactivated vaccine, and DNA vaccine is needed and rotavirus VLP vaccine is one of the promising approaches for vaccine candidate [2].

In this study, three rotavirus structural proteins are expressed in BEVS to produce rotavirus-like particles as

*Corresponding author
Phone: 82-31-280-6260; Fax: 82-31-280-6239;
E-mail: jskim3@greencrossvaccine.com

the vaccine candidate. Culture condition of *Sf9* cell in the bioreactor up to 50-l scale and VLP production condition are optimized. Biochemical and immunological properties of VLP are studied.

MATERIALS AND METHODS

Cell Line

The *Sf9* cell line was cloned by G.E. Smith and C.L. Cherry in 1983 from the parent line IPLV-SF21 AE, which was derived from pupa ovarian tissue of the fall armyworm, *Spodoptera frugiperda*, by Vaughn, *et al.* in 1977 [19]. The *Sf9* cell line, ATCC CRL-1771, was obtained from ATCC at passage 16 and a Master Cell Bank (MCB) was prepared by MAGENTA Corporation, Rockville, Maryland at passage 53. Serum free adapted *Sf9* cell was used for the preparation of MCB. Working Cell Bank (WCB) was prepared at passage 61, and was used in this study.

Recombinant Baculoviruses

Three recombinant baculoviruses expressing each rotavirus gene VP2, VP6, and VP7 (serotype G3) were obtained from Wyethe Ledele Vaccine and Pediatrics, U.S.A. VP6 and VP7 proteins are known to be essential for correctly presenting epitopes in recombinant VLP, and VP2 protein was used for maintaining a stable VLP structure. Briefly, recombinant baculovirus expressing VP2, named pVL941 G2bRf, was constructed by Estes by using pVL941 as vector and VP2 gene from bovine rotavirus RF strain [11]. BRV6 recombinant baculovirus encoding VP6 gene from bovine rotavirus C486 strain was constructed by using the pAC373 vector [6]. A partial VP7 (serotype G3) gene from simian rotavirus SA11 strain was cloned in pVL941 vector, and it was named as VL941 G9/2-1 [18]. Master Virus Seed Banks of three recombinant baculoviruses were also prepared by the MAGENTA Corporation. Working Virus Seed Banks were prepared by the GreenCross Vaccine Corporation, and were used in this study.

Sf9 Cell Culture and VLP Production

Sf9 cell was maintained in a spinner flask in 28°C. Serum and protein free media (Insect-XPRESS, BioWhittaker, Maryland) were used for the *Sf9* cell culture. For the scale-up of *Sf9* cell culture and VLP production, CelliGen Plus bioreactor [New Brunswick Scientific (NBS), New Jersey, U.S.A.] was used for the 5-l cultivation. BioFlo4500 (NBS) and BioFlo6000 (NBS) model bioreactors were used for 10-l and 50-l cultivation, respectively. Culture condition and data in the bioreactor culture were controlled and recorded by using the Biocommand 32 program (NBS). Three gases, including air, nitrogen, and oxygen, were used for the D.O. control and a pitched blade type impeller was used for agitation. The temperature was controlled at 27°C and the pH was not controlled. For the production of VLP, three recombinant baculoviruses, encoding each rotavirus VP2, VP6, and VP7, were infected to the *Sf9* cell culture with different moi. After 6 days of incubation, culture supernatant was concentrated by ultrafiltration and semipurified by ultracentrifugation (25,000 ×g, 2 h) on 30% trehalose cushion. Pellet was suspended in D.W. and used for further analysis and immunization.

SDS-PAGE and Western Blot

VLP samples were separated on 4–20% SDS-PAGE and transferred onto the nitrocellulose membrane. Rotavirus proteins were detected with a mixture of monoclonal antibodies that specifically bound to each VP2, VP6, and VP7. The amount of expressed protein was analyzed by using ImageMaster 1D Elite version 3.01 program (Amersham Pharmacia Biotech).

Electron Microscopy

Formvar-carbon-coated 200 mesh copper grids (TED PELLA, Inc.) were placed on the surface of the droplet of semipurified VLP or SA11 sample. After 30 sec, most of the solution was removed from the grid with filter paper. The grid was placed on the aqueous uranyl acetate solution for 30 sec and dried. VLP and SA11 particles were observed under a transmission electron microscope, model JEM1010 (JEOL, Japan) at NICEM (The National Instrumentation Center of Environmental Management, Seoul National University).

Immunization

Three-weeks-old ICR mice were purchased from Chareles River Corp., Japan. Rotavirus infection was monitored before immunization by rotavirus-specific ELISA. Thirty μg of the semipurified VLP was immunized intramuscularly in mice. Protein concentration was measured by BCA assay. Sera from immunized mice were collected at 2-week intervals after immunization, and rotavirus-specific antibody was titered by ELISA. Sera from immunized mice were serially diluted and added to a 96-well plate coated with rotavirus SA11 strain. After 1 h incubation, HRP-conjugated anti-mouse antibody was added and incubated for 30 min. TMB substrate was added and incubated for 30 min. Color development was stopped by addition of sulfuric acid solution, then O.D. at 450 nm was measured. The antibody titer was expressed as the reciprocal of the highest serum dilution, where the measured P/N ratio was higher than 2 and O.D. higher than 0.2.

RESULTS AND DISCUSSION

Sf9 Cell Culture in Bioreactor

Sf9 cells were normally maintained at cell density between 3.0E+05 and 3.0E+06 cells/ml in a spinner flask and

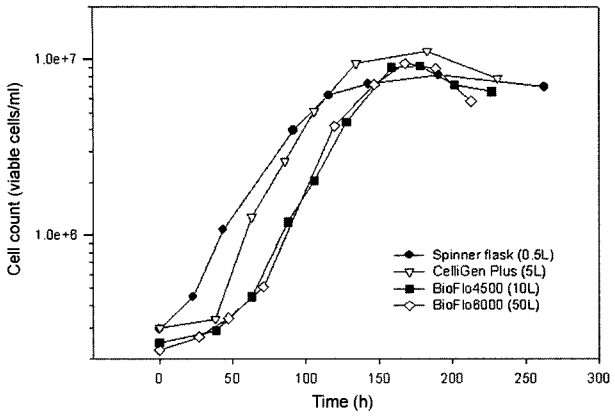

Fig. 1. Growth of *Sf9* cells in different culture volumes.
Growth of *Sf9* cell was compared in 0.5 l (●), 5 l (▽), 10 l (■), and 50 l (◇). The maximal cell concentration is higher in bioreactor (5, 10, 50 l) than spinner flask (0.5 l) culture but there is a longer lag phase in bioreactor than spinner flask culture.

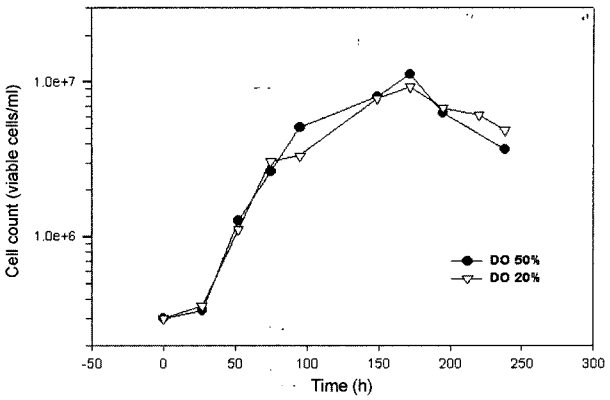

Fig. 2. Effect of dissolved oxygen (D.O.) level on *Sf9* cell growth.
Sf9 cell was grown in a 5-l bioreactor with controlled 20% (▽) and 50% (●) D.O. level. There was no significant difference in cell growth.

subcultured at 3 or 4 days interval. *Sf9* cells were grown to maximal concentration of 8.16E+06 cells/ml in a 500-ml spinner flask, and its doubling time at exponentially growing phase was 24.5 h. The culture volume was scaled up to 5, .0, and 50-l in a bioreactor, and their growth was compared. Three models of the NBS bioreactor were stirred tank with a pitched blade impeller. The maximal cell concentrations in the bioreactor were 1.12E+07 cells/ml in 5-l, 9.25E+06 cells/ml in 10-l, and 9.50E+06 cells/ml in 50-l culture. Doubling time at exponentially growing phase was 20.1 h in 5-l, 22.2 h in 10-l, and 25.2 h in 50-l culture. It was less or similar to that of the spinner flask, but the lag phase was expanded as the culture volume increased. The cell concentration in bioreactor was decreased after maximal cell concentration, compared to spinner flask culture, and it might be caused by decrease of viability (Fig. 1). The growth condition would be changed to optimally reduce the shearing force to increase viability in 10 and 50-l cultures.

The effect of different D.O. levels on the *Sf9* cell growth was evaluated in a 5-l bioreactor culture. The growth of *Sf9* cell was similar in 20% and 50% D.O. (Fig. 2) and similar results were reported from several bioreactor studies where it was shown that *Sf9* cell actually grew well in a wide range of D.O. levels [16].

Glucose consumption and lactate production were compared in the spinner flask and the 5-l bioreactor culture. Glucose concentration dropped sharply and the most glucose was consumed when the cell growth reached its maximal concentration level. Lactate was steadily accumulated in the spinner flask culture, but there was no lactate accumulation in the bioreactor culture (Fig. 3). In the bioreactor culture, D.O. was maintained at 50%, and there seemed to be enough oxygen for effective oxidation of glucose. Effective utilization of glucose as energy source would be one of the reasons for the higher cell growth in the bioreactor than in the spinner flask.

VLP Production

Exponentially growing *Sf9* cells were infected with three recombinant baculoviruses. The highest VLP expression was obtained when recombinant baculovirus were infected at the cell concentration of about 1.0- 1.2E+06 cells/ml (data not shown). Different moi of three recombinant baculoviruses were tested, and the result was compared by Western blot (Table 1). Specific moi ratio (1.0:2.0:0.2 for VP2:VP6:VP7) with the highest relative amount of VLP was used for further studies. Although there is no direct comparison data of the VLP expression in different culture scales, no significant differences were found by Western blot analysis. After infection, *Sf9* cells grew slowly and the viability decreased sharply. VLP was

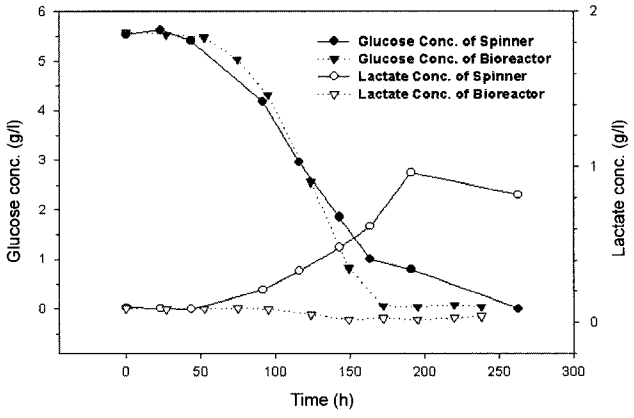

Fig. 3. Comparison of glucose/lactate concentration during *Sf9* cultivation in 0.5-l spinner and 5-l bioreactor cultures.
D.O. 50% was maintained in the 5-l bioreactor culture (▼, glucose; ▽, lactate) but not controlled in the 0.5-l spinner culture (●, glucose; ○, lactate).

Table 1. Comparison of VLP expression in *Sf9* cells infected with different moi. Three recombinant baculoviruses were infected with a different moi ratio in 0.5-l spinner flasks and the relative amount (R.A.) of expressed protein content against sample 1 were analyzed in a Western blot.

		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
VP2	moi	1.0	0.2	0.2	1.0	1.0	1.0
	R.A.	1.00	0.84	1.49	1.44	1.86	1.42
VP6	moi	1.0	0.2	2.0	1.0	2.0	2.0
	R.A.	1.00	0.97	1.11	1.11	1.29	1.16
VP7	moi	1.0	0.2	0.2	0.2	0.2	1.0
	R.A.	1.00	0.96	1.10	0.99	1.09	1.00
Total	R.A.	1.00	0.93	1.21	1.15	1.37	1.16

harvested 6 days after infection, when the viability was about 10% (Fig. 4).

The cultured VLP was clarified by centrifugation at 8,000 ×g for 20 min. VLP was semipurified from the clarified culture supernatant by ultrafiltration, and ultracentrifugation on 30% trehalose cushion. The pellet was suspended in D.W. VLP preparation was separated on 4-20% SDS-PAGE and transferred onto the NC membrane. Rotavirus protein VP2, VP6, and VP7 were detected by three monoclonal antibodies that were specific to each viral proteins (Fig. 5). Three rotavirus proteins were detected at around 94 kDa, 41 kDa, and 38 kDa molecular weight. Comparing with wild-type rotavirus SA11 (lane 2), VP7 protein of VLP was detected at the lower molecular weight of about ~36 kDa. To construct recombinant baculovirus expressing VP7, cDNA of SA11 gene 9 from bp 92 was inserted into the pVL941. This resulted in the utilization of the second initiation codon for the expression of VP7 and showed a lower molecular weight than the wild-type VP7 [18]. Three bands were also detected at the VP2 size in SA11. Many VLP lot's produced in our laboratory showed three bands of VP2 molecular weight. Several cleavage products by protease at the VP2 size have been reported [18] but only one VP2 band was detected in this experiment. VLP preparation was viewed under electron microscopy, and it showed the three-layered empty structure that was very similar to wild-type rotavirus structure (Fig. 6). VLP and wild-type rotavirus SA11 preparations contained partially broken and two-layered particles.

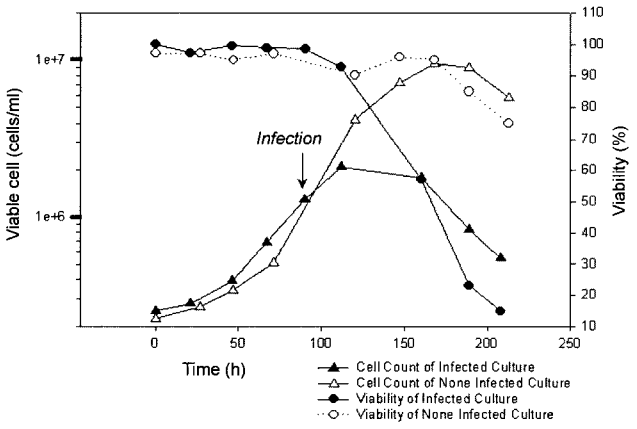

Fig. 4. Growth of *Sf9* cells after tri-infection of recombinant baculovirus for VLP production.
The viable cell concentration was compared in normal (△) and tri-infected 50-l cell culture (▲). Viability of infected culture (●) was sharply decreased after infection compared to normal culture (○).

Immunogenecity

Four-weeks-old ICR mice were immunized with 30 μg of semipurified VLP intramuscularly. PBS was similarly injected as a control. The sera were obtained from the immunized mice, and the rotavirus-specific antibody was titered as described in Materials and Method. Antibody titer reached a maximal at 6 weeks post-immunization and lasted for 21 weeks (Fig. 7). Although there was no clear

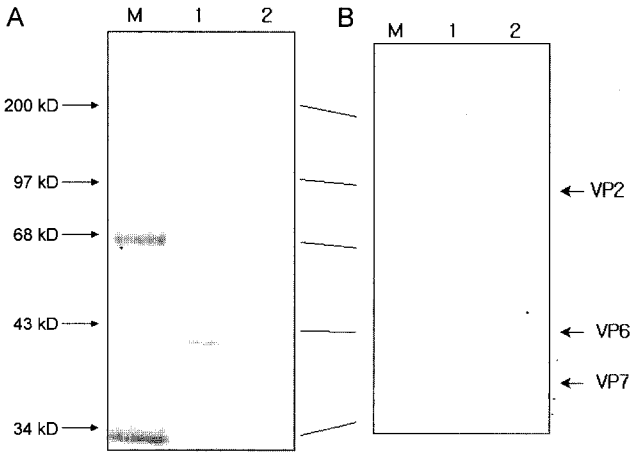

Fig. 5. Western blot analysis of VLPs.
VLPs and SA11 rotavirus were separated on 4-20% SDS-PAGE and transferred on NC membrane. VP2, VP6, and VP7 were detected with mixture solution of three monoclonal antibodies specific to each viral protein. A, Coomasie blue stain; B, Western blot; lane M, molecular size marker; lane 1, VLP; lane 2, SA11. (←) indicates VP2, VP6, and VP7 proteins.

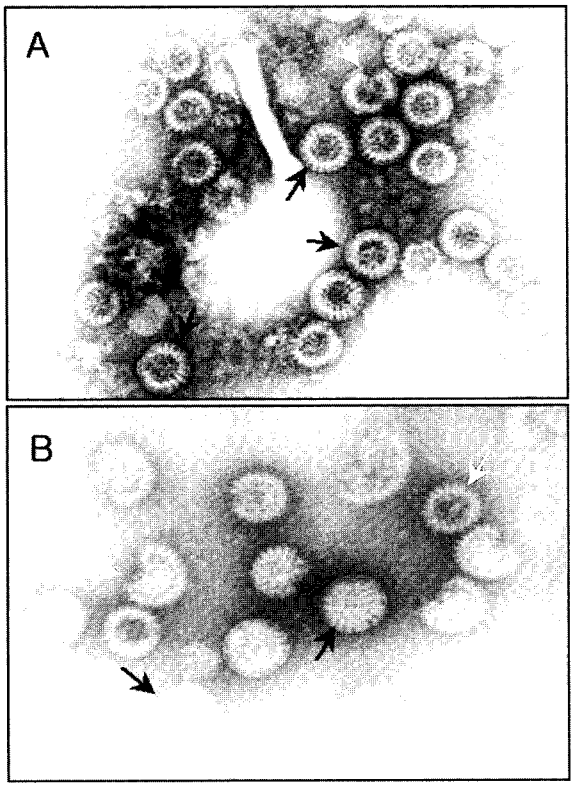

Fig. 6. Electron microscopy of VLP and rotavirus SA11 stain.
VLP (A, 80,000×) and SA11 (B, 100,000×) showed complete triple-layered structure (closed arrow). It also contained incomplete and broken particles (open arrow).

correlation between ELISA titer and protection, the induction of ELISA titer indicated its immunogenic property. Neutralizing antibody test and further challenge test should be performed to evaluate VLP as a vaccine candidate.

The U.S. Food and Drug Administration approved a live virus vaccine (Rotashield™, Wyeth Laboratories, Inc.) for the use in children in 1998. However, the Advisory Committee on Immunization Practices (ACIP) in 1999 recommended that Rotashield™ should be discontinued for infants in the United States, because data indicated a strong association between Rotashield™ and intussusception (bowel obstruction) among some infants during the first 1-2 weeks after vaccination [8]. Although there are still some arguments in its association with intussusception [3], a new safe vaccine against rotavirus is required. Several vaccine studies have been reported, including live vaccine based on bovine rotavirus or salmonella vector, inactivated rotavirus, and DNA vaccines [1, 2, 4, 14]. VLP vaccine is one of the promising approaches for the prevention of rotaviral disease. In the present study, production, purification, and immunogenecity of rotavirus VLP expressed in BEVS were conducted in a 50-l scale.

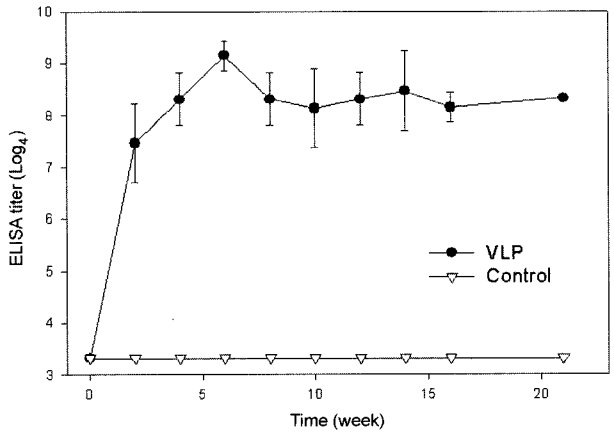

Fig. 7. Immunogenecity of VLP.
Sera from mice immunized with VLP were titered by ELISA for 21 weeks. (●), VLP-immunized mice group; (▽), control mice group.

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