Production of EV71 vaccine candidates

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Enterovirus 71 (EV71) is now recognized as an emerging neurotropic virus in Asia and with Coxsackie virus (CV) it is the other major causative agent of hand-foot-mouth diseases (HFMD). Effective medications and/or prophylactic vaccines against HFMD are urgently needed. From a scientific (the feasibility of bioprocess, immunological responses and potency in animal challenge model) and business development (cost of goods) points of view, we in this review address and discuss the pros and cons of different EV71 vaccine candidates that have been produced and evaluated in animal models. Epitope-based synthetic peptide vaccine candidates containing residues 211–225 of VP1 formulated with Freund's adjuvant (CFA/IFA) elicited low EV71 virus neutralizing antibody responses, but were protective in the suckling mouse challenge model. Among recombinant EV71 subunits (rVP1, rVP2 and rVP3) expressed in *E. coli*, purified and formulated with CFA/IFA, only VP1 elicited mouse antibody responses with measurable EV71-specific virus neutralization titers. Immunization of mice with either a DNA plasmid containing VP1 gene or VP1 expressed in *Salmonella typhimurium* also generated neutralizing antibody responses and protected animals against a live EV71 challenge. Recombinant EV71 virus-like particles (rVLP) produced from baculovirus formulated either with CFA/IFA or alum elicited good virus neutralization titers in both mice and non-human primates, and were found to be protective in the suckling mouse EV71 challenge model.

Synthetic peptides or recombinant EV71 subunit vaccines (rVP1 and rVLP) formulated in alum were found to be poorly immunogenic in rabbits. Only formalin-inactivated (FI) EV71 virions formulated in alum elicited cross-neutralizing antibodies against different EV71 genotypes in mice, rabbits and non-human primates but induced weak neutralizing responses against CAV16. From a regulatory, economic and market acceptability standpoint, FI-EV71 virion vaccines are the most promising candidates and are currently being evaluated in human clinical trials. We further describe and analyze some new bioprocesses technologies that have great potential applications in EV71 vaccine development. This review also demonstrates the opportunities and challenges that the Asian vaccine industry faces today.

Introduction

Hand-foot-mouth diseases (HFMD) are mainly caused by Coxsackie virus (CV) and Enterovirus 71 (EV71) infections and have become serious public health problems in Southeast Asia.[1-5] EV71 epidemics have always been associated with fatalities and neurological complications in children.[1-5] Several EV71 vaccine candidates are presently being developed and evaluated in human clinical trials.[3-8] EV71 is a non-enveloped RNA virus of the Picornaviridae family. The viral particle size is 30–35 nm in diameter and contains a single plus sense ssRNA molecule (7.5–8.5 kb) and four structural proteins VP1, VP2, VP3 and VP4.[9-11] VP1 has often

been used for EV71 molecular genotyping and epidemiological monitoring. EV71 is classified into 3 genotypes A, B, and C, and further divided into B1-B5 and C1-C5 sub-genotypes.[11-14] B5 and C4 isolates were recently identified in epidemics in Malaysia, Singapore, Taiwan, Thailand and China.[13,14] Based on the molecular epidemiological surveillance in Taiwan, CVA6, CVA10 and in particular CVA16 are also the most common viruses causing HFMD in children.[14] Therefore, an effective HFMD vaccine should elicit strong cross-neutralizing antibody responses against different genotypes of both EV71 and CV.

We recently reported the results obtained from animals immunized with different vaccine candidates produced using various platform technologies (Chou et al., manuscript submitted to Clinical and Developmental Immunology). These vaccine candidates include synthetic peptides containing virus neutralization epitopes, virus-like particles expressed in the baculovirus system, recombinant EV71 subunits produced from *E. coli*, and formalin-inactivated EV71 grown in Vero cell cultures. To establish correlates of protection and overcome the genetic and antigenic variations in enteroviruses, we use standardized antigens and immunological assays to compare immune responses elicited by each vaccine candidate. The results obtained from the immunogenicity studies have certainly provided valuable and critical information for selecting EV71 vaccine candidate. In this review, we further examine the regulatory issues associated with the manufacturing process, the cost of goods and the market acceptability for each vaccine prototype.

EV71 Vaccine Candidates

Criteria and factors for selecting potent EV71 vaccine candidates acceptable to regulatory authorities. EV71 and CVA16 are two major causative agents of HFMD, so effective and prophylactic vaccines should elicit immune responses neutralizing both EV71 and CVA16. Although a clinical trial of formalin-inactivated EV71 virion vaccine formulated with Al(OH)$_3$ was tested during the 1975 Bulgarian

epidemic, the detailed results of the trial were not published.[15] EV71 vaccine candidates based on chemically-inactivated virions, synthetic peptides, recombinant subunits, virus-like particles and DNA are being developed and reported in the literature.[3-7,16-21] To select potent EV71 vaccine candidates compliant with regulatory guidelines, a number of specific criteria and factors should be considered. Because of their success, live attenuated and chemically-inactivated polio vaccines should be used as references and models. The serum neutralizing antibody responses elicited by polio vaccines correlate with protection in children and life-long immunity.[22] Therefore, the first critical factor for EV71 vaccine candidates is to evaluate whether it will elicit strong cross-genotype neutralizing antibody responses in animal immunogenicity studies. Different assays are available for measuring neutralizing antibody titers, but they are not standardized and validated. Currently there is no standardized assay to quantify vaccine antigens that has been approved by regulatory authorities. Without these critical assays, it is difficult to compare the merits of different EV71 candidates and select the most potent vaccine for clinical trials. To overcome these deficiencies, we (the Vaccine R&D Center at National Health Research Institutes, Taiwan) published a series of peer-reviewed papers to describe the preparations of EV71 virus stocks; the development and qualification of an in-house standardized virus neutralization assay (RD cell micro-neutralization assay); the methodology for quantifying EV71 antigens (VP2-based Q-ELISA) in vaccine samples.[7,19-21] The immunogenicity results obtained from animals immunized with different vaccine candidates produced using various platform technologies are summarized below. The detailed results from these studies are currently submitted for publication elsewhere (Chou et al., manuscript submitted to *Clinical and Developmental Immunology*). Based on our results, we can rank the EV71 vaccine candidates according to their potency and efficacy. The vaccine potency and efficacy are defined as cross-genotype virus neutralization titer and protection in the suckling mouse challenge model, respectively.

Neutralization epitopes-based EV71 vaccine candidate. Several linear immunodominant neutralization epitopes VP1-43, VP1-49 and VP2-28 that respectively correspond to residues 211–225, 240–255 of VP1 and residues 136–150 of VP2[21,23-26] have selected to develop peptide-based EV71 vaccines. Synthetic peptides containing these epitopes had been tested in both BALB/c and C56BL/6 mice at different doses (10 to 100 μg) of either individual or mixed synthetic peptides formulated either in PBS, alum, or complete Freund's adjuvant (CFA)/ incomplete Freund's adjuvant (IFA). We and others[21,24-26] have shown that synthetic peptides formulated with CFA/IFA could induce antibody responses as determined by the peptide-ELISA and western blot analysis. VP1-43 alone or mixed with other peptides in the presence of CFA elicited low virus neutralization titers (1/32) against the vaccine EV71 E59 isolate (B4 subgenotype), while other synthetic peptides did not. Similarly Foo et al.,[24,25] reported that a synthetic peptide (SP70) containing residues 211–215 of VP1 elicited virus-neutralizing antibody response (1/32 titer) and protected newborn mice against lethal EV71 challenges by passive immunization. There is 60% homology between EV71 and CVA16 at the VP1-43 peptide sequence,[21] but VP1-43 specific antibodies failed to neutralize CVA16. So far, there is no evidence indicating that antibodies generated against EV71 peptides can neutralize CVA16. Interestingly, VP1-43 of CVA16 formulated with CFA/IFA was found to be non-immunogenic and failed to elicit anti-VP1-43 IgG responses (unpublished results). In rabbits, VP1-43 peptide formulated in CFA/IFA elicited low level of antibody responses (1/800) that had no virus neutralization activity. Fully synthetic peptides containing neutralization epitopes could (1) provide well-defined and cost-effective immunogens and safety advantages; (2) reduce any potential undesirable immune responses such as antibody-dependent enhancement (ADE) that were reported in two recent papers,[27,28] and (3) promote EV71 vaccine usage in developing countries due to the low cost of finished products, they however are intrinsically poorly immunogenic and require to be formulated in strong

adjuvant such as CFA that is not acceptable by the regulatory agencies. Thus, much more work is needed to develop efficacious synthetic peptide-based EV71 vaccines acceptable from the regulatory and commercial point of view.

Recombinant subunit-based EV71 vaccine candidates. The structural organization of the Picornaviridae suggested that the VP1, VP2 and VP3 antigens are exposed on the viral surface that has been confirmed by the recent crystallographic studies of EV71,[29] and thus could serve as targets for EV71 subunit vaccine development. To compare the immune responses they elicit in mice, rVP1, rVP2 and rVP3 were produced, purified and formulated with or without alum. Mouse antisera were found to recognize the respective proteins on western blots, but none of them could neutralize EV71 virus in vitro. However, in the presence of CFA/IFA, recombinant VP1 was the only subunit capable of eliciting antibody responses with EV71 neutralization titers of 1/128 (ref. 16, our unpublished results). In addition, no cross-genotype virus neutralizing activity was detected [our unpublished results]. In rabbit immunogenicity studies, rVP1 formulated in CFA/IFA did elicit strong antibody responses, but had no EV71 virus neutralizing activity. Chiu et al.,[30] also reported that rVP1 expressed in *Salmonella typhimurium* and purified for vaccine formulation with CFA/IFA also induced EV71-specific neutralizing antibody responses and protected sucking mice in a live virus challenge model study. All rVP1-specific antisera failed to neutralize CVA16. Recombinant antigen-based EV71 vaccines are easy and cheap to manufacture, but they require CFA (non-FDA approval adjuvant) and elicit low neutralization titers. Unless new acceptable adjuvant can significantly enhance the immunogenicity of EV71 subunit vaccines, they are currently not the first choice for evaluation in phase 1 human clinical trials.

Genetic vaccines have great potential for vaccine development since their gene products are expressed intra-cellularly like during natural viral infection and thus can stimulate both humoral and cellular immunity. Mouse immunogenicity studies of EV71 DNA vaccine harboring the VP1 gene have been reported by Wu et al.,[16] and Tung et al.[18] VP1-based DNA vaccine candidates could elicit low levels of mouse neutralizing antibody responses at high doses (100 μg of DNA plasmid). Currently, DNA vaccine has not been able to elicit strong antibody responses in humans. No human DNA vaccine has been so far licensed, and significant improvements are necessary before a genetic EV71 vaccine becomes a valuable option. Low levels of EV71-specific neutralizing antibodies (1/32 titer) could be generated by oral immunization of mice using transgenic tomatoes expressing the VP1 protein.[31] Edible vaccines had been proposed to be the next wave of human vaccines for developing countries. Beside the poor immunogenicity of edible vaccines in humans, the regulatory issues will most likely a major hurdle for them to be licensed and used in humans, since their stability will not be last over two months (can a banana or tomatoes or corn last more than a month?). In addition, the conditions for the growth of plants in the field and the consistent harvest time of fruits could be difficult for GMP validation.

VLP-based EV71 vaccine candidate. VLP-based prophylactic vaccines have been successful against hepatitis B virus and human papillomavirus and are now commercially available,[32] VLP-based EV71 (VLP-EV71) vaccines produced from recombinant baculovirus could be good vaccine candidates.[10,33] In our laboratories, vaccination of young mice (6 to 8 weeks old) with 5 μg of VLP-EV71 alone or in the presence of either alum or CFA/IFA produced antibodies with virus neutralization titers of 1/64, 1/128 and 1/160, respectively. Chung et al.,[33] had reported that 10 μg of EV71 VLPs formulated with CFA could elicit virus neutralizing antibody responses with a titer of 1/512 in mice and protected newborn mice against lethal EV71 challenges by passive immunization. These mouse antisera could neutralize different EV71 genotypes (C2, B4, C4, B5 and C5).[33] In both rabbit and non-human primate immunogenicity studies, only high dose (> 100 μg) of VLP-EV71 formulated with alum could induce virus neutralizing antibody responses [our unpublished results]. In addition, these EV71-specific antisera failed to neutralize CVA16. Although these results suggest that VLP-EV71 may mimic the structural organization of EV71, one may still need high doses of vaccine to optimize mimicry in order to elicit antibodies capable of cross-neutralizing CVA16. The time needed for recombinant baculovirus VLP-EV71 constructs and insect cells to be GMP certified, and the cost of VLP-EV71 vaccine production (> 100 μg/dose) would not allow a VLP-EV71 vaccine candidate to enter phase 1 clinical trials in the near future.

Formalin-inactivated EV71 virions as vaccine candidates. A potential live-attenuated EV71 vaccine candidate (S1–3') developed by the Shimizu's group in Japan[34] had been shown to elicit a broad spectrum of neutralizing antibody responses against different genotypes of EV71 in cynomolgus monkeys, but mild neurological symptoms were also observed. The concern over potential reversion of live-attenuated vaccine to virulence[35,36] has made chemically-inactivated virion-based vaccines a more attractive choice for EV71 vaccine development. We have evaluated the potency of formalin-inactivated EV71 virion based on a B4 subgenotype isolate produced in Vero cells cultured in either serum-containing or serum-free medium in different animal immunogenicity studies.[7,19,37] Formalin-inactivated EV71 virion (EV71vac) was found to induce superior immune responses compared with the other vaccine prototypes described above. EV71vac elicited mouse antibody responses that cross-neutralized different EV71 subgenotypes (B1, B3, B4, B5, C2, C4 and C5) with neutralization titers ranging from 1/64 to 1/1,280, but failed to neutralize CVA16. In contrast, antisera generated from either rabbits or non-human primate macaques immunized with the same amount (2 μg) of EV71vac formulated with alum strongly cross-neutralized different EV71 subgenotypes and weakly cross-neutralized CVA16, with average titers of 1/6,400 and 1/32, respectively. Similar results were reported by both Bek et al. and Dong et al.[38,39] who produced formalin-inactivated EV71 vaccine candidates based on a C4 genotype virus. These studies also revealed that these vaccine candidates were highly immunogenic

Table 1. Formalin-inactivated EV71 whole-virus vaccine candidates currently being tested in clinical trials

| Organizations | Manufacturing processes | | | | Clinical trials | | Reference (clinicaltrials.gov Identifier) |
	Cell lines and EV71 strain	Up-stream	Down-stream	Dosage (μg of EV71 antigen)	Population target	Current status	
NHRI[a] (Taiwan)	Vero cell and EV71 B4 subgenotype (GMP-certified)	Roller-bottle (Serum-free media)	Gel-filtration Chromatography	5 and 10	Young adults	Phase 1 completed	NCT01268787
Sinovac (China)	Vero cell and EV71 C4 subgenotype	Cell factory (Serum-free media)	Gel-filtration Chromatography	0.25, 0.5 and 1[c]	Young adults, young children and infants	Phase 1 and 2 completed Phase 3 now	Li et al.,[8] NCT01273246, NCT01273233 NCT01507857
Beijing Vigoo (China)	Vero cell and EV71 C4 subgenotype	Cell factory (Serum-free media)	Gel-filtration Chromatography	0.4, 0.8 and 1.6	Young adults, young children and infants	Phase 1 and 2 completed Phase 3 now	NCT01313715, NCT01399853 NCT01508247
CAMS[b] (China)	Human diploid cell KMB-17 and EV71 C4 subgenotype	Cell factory (Serum-containing media)	Gel-filtration Chromatography	Unknown[d]	Young adults, young children and infants	Phase 1 completed Phase 2 now	NCT01391494, NCT01512706
Inviragen (Singapore)	Vero cell and EV71 B3 subgenotype	Cell factory (Serum-free media)	Gel-filtration Chromatography	0.3 and 3	Young adults,	Phase 1 completed	NCT01376479

[a]NHRI means National Health Research Institutes, Taiwan. [b]CAMS means Chinese Academy of Medical Sciences, Kunming, China. [c]The antigen dosage is calculated based on the report by Liang et al.,[46] that the specific activity of EV71 antigen reference standard is established in China to be 421.1 U/μg. [d]The specific dosage used in these clinical trials by CAMS was not reported.

and could elicit cross-genotype neutralizing antibody responses in mice and non-human primate models. Taking these results together, we can conclude that the potent and important cross-genotype neutralization epitopes of EV71 virus are conformational and might not be mimicked by synthetic peptides or recombinant subunit antigens. Furthermore, the current results suggest that common neutralization epitopes (most likely conformational) exist in EV71 virus and contribute to eliciting strong antibody responses that are capable of cross-neutralizing different EV71 genotypes. In addition, the amino acid sequence dissimilarity between EV71 and CVA16 can partially explain the observation that mouse antibodies failed to cross-neutralize CVA16 (which may have similar structural organization like EV71). From our preliminary results, formalin-inactivated CVA16 virion (CVA16vac) could not elicit mouse cross-neutralizing antibodies against different EV71 genotypes. Therefore, based on current information the best formulation for producing a stable and cost-effective of HFMD vaccine is a combination of EV71vac and CAV16vac.

Product Development of Cell-Based Formalin-Inactivated EV71 Vaccines

Since HFMD mainly threatens the children in Asian developing countries, an ideal EV71 vaccine should be inexpensive, safe, easy to administer and acceptable to most parents. As mentioned above, inactivated EV71 virions are the most promising EV71 vaccines and could be licensed by regulatory agencies sooner than any other EV71 vaccine candidates. Five institutes (3 profit-orientated companies and 2 government-supported non-profit organizations) have produced inactivated EV71 virion vaccines based on different EV71 vaccine strains using different manufacturing technologies and completed human phase 1 clinical trials in China, Taiwan and Singapore (**Table 1**). Since vaccine companies from developed countries have little incentive to develop EV71 vaccines and only a few vaccine companies in the Asia-Pacific region have capability to take a vaccine from research to product launch, more effort and cooperation between these five institutes is urgently needed. The following sections provide important

information about the vaccine manufacturing processes that should be shared and discussed.

Vaccine strain selection and characterizations. The identification and selection of an EV71 vaccine strain which can grow to high titers in Vero cell (FDA approved cell line) and induce cross-genotype virus neutralizing antibody responses represent the first step in EV71 vaccine development. Based on US FDA and International Conference on Harmonization (ICH) guidelines, (www.fda.gove/BiologicsBloodVaccines/GuidanceCompliance Regulatory Information/Guidances/default.htm and ICH documents Q5A and Q5D), we have recently described the characterization and validation of a clinical isolate E59 belonging to B4 sub-genotype based on VP1 genetic analysis.[19] The E59 vaccine strain has shown strong genetic stability during passages based on the nucleotide sequences obtained from the master virus seed (MVS), working seed banks (WVS) and virus harvested from the production lots, and that have been found to be identical to those of the original isolate. Compared with published sequences of

the genotype A BrCr strain,[19] VP4, VP2, VP3 and VP1 of the E59 strain have 26.8, 35.8, 18.5 and 35.7%, respectively nucleotide differences with the corresponding gene segments of the BrCr strain. In contrast, the amino acid sequences in viral proteins between the BrCr and E59 virus were found to be 0, 1.6 (4 amino acids different), 2.1 (5 amino acids different) and 6.7% (20 amino acids different) difference respectively. Amino acids changed at position 163 of VP2 (Pro to Gln; position 557 of VP3 (Asp to His); and positions 587, 595, 663, 710, 739, 809 and 811 of VP1 (Pro to Gln, Pro to Gln, Lys to Glu, Arg to Gln, Pro-Gln, Glu to Lys and Ser to Pro, respectively) could significantly alter the global structure of the viral capsid and cause the virus to switch from the A to the B genotype.

Different inactivation methods were investigated to optimize the down-stream bioprocess.[19] These included formalin-inactivation at 37°C for 3 d, exposure to UV light (UV box equipped with a 3,600 Å light source) for 10 min at room temperature, heat-treatment at 65°C for 2 h, and cross-linking with 0.05% (v/v) glutaraldehyde for 6 weeks at 4°C. Based on results after two passages in eggs and virus infectiousness, all methods were found to be very efficient at inactivating EV71 virus in culture supernatants, but significant material precipitation was observed in heat-treated and glutaraldehyde-inactivated EV71 samples. Thus, rabbit immunogenicity studies were performed with formalin-inactivated and UV inactivated EV71 preparations. The neutralizing antibody titers obtained with UV-inactivated EV71 vaccine formulations were found to be lower than those obtained with the formalin-inactivated vaccines.[19]

Qualification of cell substrates and virus seeds. Currently, the global vaccine manufacturing capacity is concentrated in Australia, Europe and North America and companies from these countries have little incentive to produce EV71 vaccines due to their low market value estimated to be 50 to 80 million US dollars per year (excluding China market) in 2005. Egg-based and cell-based culture systems are two mature technologies for manufacturing viral vaccines. The egg-based technology has a long success history at supplying

seasonal influenza vaccines but it will be a big investment for developing countries to build and validate GMP egg-based manufacturing facilities in a short period time. Furthermore, developing countries may have significant problems with qualified-egg supplies. Therefore, cell-based technologies are becoming an attractive option to establish new manufacturing facilities in developing countries. Currently only a few cell lines (Vero, MDCK, MRC-5) are GMP-certified for vaccine productions. Based on our experience, EV71 and CVA16 did not replicate well in cells grown in suspension. The Vero cell line is the most popular continuous cell line for manufacturing human vaccines and Vero cell banks fulfilling the cGMP requirements are readily available from American Type Culture Collection (ATCC). In addition, national authorities may support the establishment of cell-culture based manufacturing facilities that can also be used for emergency influenza pandemic vaccine production. Thus, the worldwide production of pandemic influenza vaccines could be rapidly increased to meet urgent demands during future influenza pandemics.

In order to pass WHO vaccine qualification, both the master cell bank (MCB) and MVS should be prepared and fully characterized according to US-FDA guidelines (www.fda.gove/BiologicsBloodVaccines/ GuidanceCompliance Regulatory Information/Guidances/default.htm). The MCB (Vero cell) and MVS (EV71 E59 strain) established at NHRI have been tested by BioReliance (UK) and found to contain no retroviruses, bovine and porcine viral contaminants and other adventitious agents according to US FDA guidelines.[19] In addition, Vero cells from MCB and virus from MVS were fully characterized and certified to not produce palpable tumors during an observation period of several weeks following inoculation into athymic nude mice. Both the WCB and WVS passed the sterility, mycoplasma, and in vivo and in intro adventitious agents tests.

Up-stream process development. Many cell-based inactivated vaccines such as polio, influenza and hepatitis A are readily available on the open market, but limited information is available on their

manufacturing processes and culture systems. Because of intellectual property (IP) rights and proprietary technologies (technical know-how) used to produce these vaccines, information on how viruses and product yields are influenced by the compositions of culture medium and the production systems used is totally missing. Serum is required for optimal growth of mammalian cells as it is a source of nutrients, hormones and growth factors. These serum factors also facilitate the attachment and spreading of cells, and provide protection against mechanical damage and shear forces.[40,41] Besides these advantages however, serum-containing (SC) medium may contain unwanted contaminants which are primary concerns for the safety of biological products.[42] To this end, the sources of bovine serum should be selected from BSE-free cattle from Australia.

Since large amounts of serum proteins in the cell culture medium could make downstream purification significantly more difficult, virus production from cell culture grown in serum-free (SF) medium is now preferable.[43] The performance of different media for Vero cell growth was screened and evaluated based on consistent cell growth performance over several passages.[7] We had tested different commercially-available SF media (Plus Vero, VP-SFM, HyQ and ExCell) and one SC medium (DMEM with 5% FBS supplement) in 75 cm[2] flasks over three passages after inoculation. We found that the average cell counts in SC medium, VP-SFM and Plus Vero was higher than those obtained with the Excell and HyQ SF media.[7] We further examined the consistency of VP-SFM and Plus Vero SF media for Vero cell growth, and found that three batches of VP-SFM yielded similar cell growth profiles, whereas culture in Plus Vero medium showed greater fluctuations and sometimes lower cell growth rates. Our results indicated that the VP-SFM medium consistently supported cell growth and high virus yields and thus was selected as serum-free medium for vaccine production.[7,37]

Currently, roller-bottles, cell factories and microcarrier bioreactors are available for up-stream cell-culture manufacturing bioprocesses. Both the roller-bottle and cell factory technologies are easy to

implement and operate, but labor intensive. Developing countries looking for a fast implementation of production infrastructure and flexibility for multiple applications, can consider to starting with either roller-bottle or cell factory technologies. In fact, the vaccine lots used in the current phase 1 clinical trials performed by the five institutes were produced using either roller-bottle or cell factory technology. To produce a million doses of EV71 vaccine (based 1 μg viral protein/dose) for immunizing Taiwanese children annually, we just need to run two-shifts with 50 L/batch using roller-bottles in a 7,500 sq. ft GMP-certified manufacturing Pilot Plant at NHRI.

If the manufacturing scale was > 2 million doses annually, the micro-carrier bioreactor technology should be considered and implemented, but assembling a skillful technical staff, and validating a manufacturing infra-structure and facility will require expensive investments (20 to 35 million US dollars). Almost every Asian government is looking to Biotechnology for food supplies and health care improvement. Some even think about achieving economic growth through the manufacture of valuable biological products. Although companies and government agencies in Asia (such as Taiwan, China, Korea and India) are progressively closing the gaps in regulatory compliance in manufacturing biologics, most vaccine companies in these countries are still behind in vaccine R&D and production techniques due to lack of financial support and technology transfer from foreign competitors. Emerging infectious diseases (SARS, pandemic avian influenza, HFMD, etc.) and potential bioterror attack have raised new challenges and have changed the landscape of vaccine industry. New technology likes "Wave" developed by Wave Biotechnology (GE Healthcare), "TideCell" by CESCO Bioengineering Co (Taiwan), and single-use bioreactors by Hyclone (US) and Sartorius Stedim Biotech (Germany) provide easy start-up and scale up disposable cell culture technology. Although there is no product manufactured by these new technologies yet, they are gaining more popularity in R&D, and pilot manufacturing process development. The

other method to increase cell growth is using micro-carrier beads that give cells more growing areas. In our own experience,[17,37,42,43] we can increase cell growth by 10-fold using a perfusion technology. Our current preliminary results indicate that we could generate 200 L of virus culture from a 50-L bioreactor using the perfusion bioprocess with serum-free medium and 5 g/liter micro-carrier, and still have virus titer about 1×10^7 TCID$_{50}$/mL (ref. 37, Liu et al., unpublished data). Other technologies have been recently developed to improve virus yields. One promising approach is to transfect more viral receptor genes into the host cell, to increase virus infection at lower MOI. The other method is to remove the genes inhibiting viral replication within the host cell by gene-knockout. Reverse-genetic (RG) technology has also been applied to improve virus yield by inserting specific protease cleavage sites or engineering temperature-sensitive growth mutants. All these improvements in virus production should significantly increase cell-culture-based EV71 vaccine production.

To avoid large amounts of host cell debris which could increase the difficulty in virus downstream purification; EV71 was collected from the culture supernatant of each bottle at the 5 d-post-infection (DPI) before the occurrence of significant cytopathic effects (CPE).[7,19,37] Cell debris could be efficiently removed by filtration through a 0.65-μm membrane. The up-stream process was investigated and optimized, and the crude virus bulk was 20-fold concentrated using either 100- or 300-kDa cut-off dia-filtration membranes in tangential flow filter (TFF) cassettes.[7,17,37] The purpose of the TFF process was to concentrate viral particles and remove cellular proteins. In most cases, more than 50% of total protein was removed. The current results suggest that some of the EV71 viral proteins not assembled in virus particles are also removed from the virus bulk during the TFF step and when infectious particles are concentrated. With an improved filter membrane set up, the whole dia-filtration and concentration processes could be fully automated in future.

Downstream process development and optimization. The best method to

improve the production cost is a simple efficient downstream purification step. Two methods are currently used to purify virus particles for vaccine production: gel-filtration chromatography and sucrose-gradient ultracentrifugation. The AKTA Pilot liquid chromatography system (GE Healthcare) equipped with Sepharose Fast Flow 6 gel was found to be very efficient at separating and purifying EV71 virus particles from pilot-scale virus concentrates (~1 L).[7,19] The EV71 virus was generally identified and located in the void volume as determined by SDS-PAGE, western blot, and TCID$_{50}$. The EV71 virus fractions were pooled and concentrated using a 100 kDa TFF membrane. The overall recovery yield based on VP2 content determined by a Q-ELISA[20] varied from 30 to 50% in six different pilot runs. Gel-filtration chromatography removed > 95% of potential protein contaminants (host residual proteins). Using Vero cell-specific ELISA, it was found that residual Vero cell proteins in virus bulks represented less than 5% of total proteins. Thus, there is still room for improvement in the chromatographic purification process.

When the formalin-inactivated viral stock was purified using sucrose gradient ultracentrifugation as previously described,[7,37] two kinds of EV71 virus particles were detected by the TCID$_{50}$ assay coupled with western blot analysis. Defective and infectious particles were found in the fractions containing 25–28% and 35–38% sucrose, respectively. When protein concentrations in pooled EV71 particle fractions were measured by the BCA method, it was found that there were three times more defective particles than infectious particles. The purified infectious EV71 viruses contain all four structural proteins VP1, VP2, VP3 and VP4. In contrast, in the defective viral particles VP0, VP1, VP3 and some VP2-associated high-molecular weight protein bands were found to be the major components as shown by SDS-PAGE and western blot analyses. Although ultra-pure EV71 infectious virus bulks could be obtained using sucrose gradient ultracentrifugation, the recovery yield was less than 20% of the chromatographically-purified viral stock. Since the defective particles had been shown to be immunogenic and to elicit

strong neutralizing antibody responses in both mice and rabbits as previously reported,[7,19,20,37] it was therefore decided that virion downstream purification would be performed using gel-filtration chromatography.

Stability profiles of vaccine bulks and formulated vaccine preparations. The stability profile of EV71 vaccine bulks based on protein assay and VP2 Q-ELISA[20] is excellent. After 1 y of storage at either 4 or 25°C, there was no sign of protein loss in the vaccine bulk.[7] Based on western blot analyses using two different monoclonal antibodies, N16 specific for the N-terminal peptide of VP1 and MAB979 specific for VP2, there was no EV71 VP1- or VP2-specific protein degradation (no protein fragments with MW below either VP1 and/or VP2 were observed) in vaccine bulks stored at 4°C for 18 mo. Mouse immunogenicity studies with vaccine bulks stored at 4°C for 0, 1, 2, 3, 6, 9 and 12 mo (2 × 2 μg/dose immunization at 2 weeks apart) revealed that these samples were highly immunogenic and elicited similar geometric mean titer (GMT) of virus neutralization against the E59 vaccine strain ranging from 200 to 600. No contamination was observed in sterility tests. Together, these results indicate that the EV71 vaccine bulks produced with the current manufacturing process and stored at 4°C for 1 y are sterile, stable, and immunogenic in mice.

When the vaccine products were analyzed using a Hitachi H-7650 electron microscope, some free inactivated EV71 particles were observed and not absorbed onto the alum. When the kinetics of viral antigens absorption to alum was examined, > 50% of viral proteins in the EV71 vaccine product was absorbed within 1 h after mixing the vaccine bulk with the aluminum phosphate at room temperature. The current data from 3 and 6 mo storage also confirmed that the rate of alum absorption is more efficient at 25°C as compared with those obtained from 4°C. In addition, it seems that the virus neutralization titers elicited in mice immunized with the EV71 vaccine correlated with the percentage of protein absorbed to alum.[7] There were no obvious losing IgG and virus neutralization titers in mouse immunogenicity studies. In the current 18-mo

stability program, the mouse immune responses also revealed that the EV71 vaccine was highly stable during long-term storage at 4°C.[7] Finally, no contamination was observed in sterility tests performed at 0, 3, 12 and 18 mo.

Preliminary results of human clinical trials. The urgent need for safe and effective vaccines against EV71 infections during outbreaks has prompted five human clinical trials that have been or are currently conducted in mainland China, Taiwan and Singapore (**Table 1**). The objective of these five randomized, double-blind and/or open-label phase 1 clinical studies is to evaluate the safety, reactogenicity and immunogenicity of formalin-inactivated EV71 vaccine candidates with dosage ranging from 0.3 to 10-μg of EV71 antigen per dose. In these trials, different numbers of eligible healthy children and/or adults were recruited and immunized with 2 to 3 doses of vaccine either 14, 21 or 28 d apart. Individual safety data were collected before and after each vaccination. Blood samples were obtained on day 0 and a day before immunization for antibody response analysis. Our preliminary clinical trial results that will be published elsewhere have indicated that safety, reactogenicity and immunogenicity of all doses (5- and 10-μg dose) were very similarly. Li et al.,[8] had also reported good safety and no reactogenicity at 0.25, 0.5 and 1 μg dose. So far all vaccine candidates had been shown to be safe and well tolerated. Our preliminary results and data from Li et al.,[8] indicate that more than 50%, 30% and 10% of the participants had pre-vaccination neutralization titers (Nt) > 8 against the vaccine strains in young adults, young children (2 to 5 y old) and infants (6 to 24 mo), respectively. Pre-existing Nt found in general populations were previously reported.[8,14,44-47] After the first EV71vac immunization, all participants seroconverted as judged by a > 4-fold increase in anti-EV71 IgG titer and > 4-fold increase in Nt was also observed in 95% of immunized young adults, but there was no further increase in Nt after the second dose (our unpublished results). The current results reported by Li et al.,[8] also indicate that two immunizations are required to achieve a 100% seroconversion with a > 4-fold increase in Nt in young

children. These results indicate that EV71 vaccine candidates are safe and efficient at eliciting neutralizing antibody responses against vaccine strains and thus providing a strong rationale for their evaluation in phase II clinical trials. In fact, two vaccine candidates produced by Sinovac and Beijing Vigoo Biological Co., Ltd. are currently being evaluated in phase 3 efficacy trials in China (ClinicalTrials.gov identifier: NCT01507857 and NCT01588247, respectively). Most likely within a year or two, we will know the efficacy and safety of formalin-inactivated EV71 vaccines based on subgenotype C4 strains.

Conclusions and Perspectives

In the past there were no standardized antigens or immuno assays to assess the potency of EV71 vaccine candidates and it has been difficult to compare which development method had yielded the vaccines which elicited the best protective immune responses. Now, using in-house standardized viral antigens and immunological assays (RD cell micro-neutralization assay), the immunogenicity results obtained from different animal studies performed with different vaccine candidates produced using various platform technologies can be compared. We can rank the EV71 vaccine candidates in the following order according to their potency (cross-genotype virus neutralization titers) and efficacy (mouse protection model): formalin-inactivated virion > recombinant virus-like particles > recombinant VP1 > synthetic peptides containing the EV71 neutralization epitope (VP1-43). Current results[7,38,39] have shown that (1) formalin-inactivated EV71 vaccine candidates based on either B3, or B4 or C4 subgenotype virus were highly immunogenic and could elicit cross-genotype neutralizing antibody responses in mice, rabbits, non-human primates and young adults; (2) the potent and important cross-genotype neutralization epitopes of EV71 are conformational and cannot not be mimicked by synthetic peptides or recombinant subunit antigens; (3) EV71 genotypes and subgenotypes share common neutralization epitopes (most likely conformational) that are capable of eliciting cross-neutralizing antibodies; and (4) the amino acid

sequence dissimilarity between EV71 and CVA16 could partially explain the observation that anti-EV71 antibodies failed to cross-neutralize CVA16 (which may not have the exact structural organization like EV71). Therefore, based on current information, a combination of formalin-inactivated EV71 and CAV16 virions may be necessary for a cost-effective bivalent HFMD vaccine.

The current clinical studies (**Table 1**) have shown that the pilot-scale manufacturing process of cell-based formalin-inactivated EV71 vaccine candidates has been successfully developed, optimized, characterized, and quantified. In the upstream process development, serum-free medium could be the optimal medium for Vero cell growth and EV71 virus production. The cell-factory and roller-bottle technologies are very efficient to produce 40 to 50 L virus culture and could be easily implemented in most Asian countries. Gel filtration chromatography was found to be the most cost-effective for downstream purification process, resulting in overall virus yields as high as 50%. The formalin-inactivated virion vaccine candidate was found to be stable > 18 mo at 4°C. At microgram-levels, viral proteins formulated with alum adjuvant could induce strong cross-genotype virus-neutralizing antibody responses in mice, rats, rabbits, non-human primates and young adults. Based on 7 batches of 40 L pilot-scale production runs, we can produce 50,000 doses of the EV71 vaccine candidate at 1 μg EV71 antigen per dose and the cost is calculated to be 0.4 US dollar/dose. Using our current GMP facility (7,500 sq ft), 1 million doses can be easily produced annually. Lee et al.,[48] has recently forecasted the economic value of an effective EV71 vaccine that could be sold at $50 or $75 US dollar per dose. With this profit margin and the new emerging vaccine market in China, the global vaccine companies now may have some interest in manufacturing EV71 vaccine.

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