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Review

Enterovirus 71 vaccine: close but still far

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SUMMARY

Background: Enterovirus 71 (EV71), a member of the *Enterovirus* genus of the *Picornaviridae* family, is one of the causative pathogens of hand-foot-and-mouth disease (HFMD) and the most common etiological agent isolated from HFMD patients complicated with neurological disorders. EV71 has become an increasingly important neurotropic enterovirus in the post-poliomyelitis eradication era. Effective antiviral agents and vaccines against this virus are currently still under development. We reviewed publications on the development of EV71 vaccines in order to provide an overview of the field.
Methods: Fifty-five articles on EV71 vaccine development, published from 1974 to 2009, were collected from Sun Yat-sen University library and reviewed.
Results: Various types of vaccine have been developed for EV71. In results published to date, all vaccines for EV71 under development appear to elicit an immune response in rodents or in monkeys. According to the established regulatory standards, it may be relatively easy to acquire a license to use the inactivated virus in order to meet the immediate demands for EV71 control . With regard to the attenuated vaccine, it is critical to increase the genetic stability before clinical use, due to the risk of virulent revertants. The virus-like particle (VLP) vaccine, not only conserving the conformational epitopes, but also having no risk of virulent revertants, is another promising vaccine candidate for EV71, but needs further development. The VP1 capsid protein is the backbone antigen protein for developing subunit vaccine and epitope vaccine; these remain viable potential vaccine strategies worthy of further study and development.
Conclusions: The conservation of the three-dimensional structure is important for the EV71 inactivated vaccine and VLP vaccine to induce a strong immune response. To develop EV71 vaccines with a high protection efficacy, strategies such as the use of adjuvant, strong promoters, tissue-specific promoters, and addition of mucosal immune adjuvant should be considered.

1. Introduction

Enterovirus 71 (EV71), a member of the *Enterovirus* genus of the *Picornaviridae* family, is the most frequently detected pathogen in hand-foot-and-mouth disease (HFMD) patients complicated with neurological dysfunction.[1] EV71 was first isolated in California in 1969,[2] and its association with HFMD was verified in 1974.[3,4] It was later confirmed as the causative agent responsible for HFMD outbreaks in Hungary,[5] Australia,[6] Hong Kong,[7] Taiwan,[8] Japan,[9] and Singapore.[10] Moreover, in 2008 and 2009, a large outbreak occurred in Mainland China.[11–13] Children under 5 years of age have been found to be particularly susceptible to the severest form of EV71-associated neurological disease.[14] This is an important public health problem causing serious clinical illness and, potentially, death in young children.

EV71 possesses a single-stranded RNA genome of approximately 7500 nucleotides, consisting of a single open reading frame (ORF) flanked by 5′-untranslated regions (5′UTR) and 3′-untranslated regions (3′UTR). The ORF is expressed as a large polyprotein that can be cleaved into P1, P2, and P3 regions. The P1 region encodes four structural proteins VP1, VP2, VP3, and VP4. The P2 and P3 regions encode nonstructural proteins, such as proteases 2A, 2B, and 3CD, responsible for virus replication and virulence. Protease 2A autocatalytically cleaves P1 at its N-terminus and liberates P1 from the nascent polyprotein,[15] while protease 3CD cleaves the P1 precursor into VP1, VP3 and VP0 (VP2 and VP4). These three structural proteins spontaneously assemble and form the crystalline virus-like particles.[16]

Though there has been a significant increase in EV71 epidemic activity throughout the Asia-Pacific region, effective antiviral therapies and vaccines have, to-date, not been available. The development of effective vaccines is a top priority in terms of control strategies. Below is an overview of the field of EV71 vaccine preparation to date.

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2. Inactivated virus vaccine

As conventional vaccines, inactivated virus vaccines, such as inactivated influenza vaccine[17] and inactivated hepatitis A vaccine,[18] have been successfully used in the human. Sero-epidemiologic studies have indicated that the preexisting neutralizing antibody to EV71 is protective against the severe outcomes of infection.[8,19] Yu et al.[20] and Wu et al.[21] showed that passive transfer of serum from formalin-inactivated and heat-inactivated virus vaccine immunized adult mice, could provide protection against EV71 challenge in neonatal mice; meanwhile, maternal immunization with inactivated EV71 vaccine was able to prolong the survival of suckling mice after EV71 lethal challenge. These results show the value of the inactivated virus vaccine for the effective control of EV71. However, the conservation of the three-dimensional structure is important in order to induce a strong immune response. Therefore, for the heat-inactivated virus, a much higher dose of viral antigen and adjuvant are required to achieve an acceptable level of immunogenicity and protection.

Obviously, an ideal vaccine strain is required for the large-scale preparation of the inactivated EV71 vaccine, as has been the case for the Sabin oral polio vaccine (OPV) strain. Lin et al.[22] developed an EV71 strain, YN3-4a, exhibiting a rapid growth rate in Vero cells with a larger plaque size and a lower lethal dose $(LD)_{50}$ in newborn mice. Lin and coworkers showed that mouse antiserum raised against YN3-4a was able to neutralize a broad range of EV71 strains isolated from patients of a variety of geographic origins at different points in time. YN3-4a possesses desirable features, such as a high viral yield, the ability to propagate in serum-free medium, and strong immunogenicity, as well as broad-based antigenic coverage and passage stability, indicating its potential for development as an inactivated vaccine strain.

A powerful cell system is also important in the development of an inactivated vaccine. As shown by Wu and coworkers,[23] a serum-free Vero cell culture with a 5 g/l Cytodex 1 microcarrier concentration in a 2-l bioreactor has also been established, yielding a high titer of 5.8×10^7 $TCID_{50}$/ml EV71 production. On the basis of the study of Wu et al.,[23] Liu et al.[24] showed that the serum-free culture increased post-infection cell death and reduced the virus productivity, but elicited a higher neutralizing antibody titer in immunized mice as compared to the serum-containing cultures. Therefore, the serum-free microcarrier culture is a valuable technique for developing inactivated EV71 vaccines on a large scale.

3. Attenuated strain vaccine

An example of a successful attenuated strain vaccine is Sabin OPV. This was introduced in the early 1960 s due to its easier administration, lower cost, and higher intestinal muscosal immunity than the inactivated polio vaccine (IPV), and has since been approved for worldwide application for poliovirus eradication.[25,26] Because of the similarities between poliovirus and EV71, Arita et al.[27,28] have developed an EV71 attenuated strain, EV71 (S1-3′), carrying mutations in the 5′UTR, 3D polymerase ($3D^{pol}$) and 3′UTR 5′ non-translated based on the attenuation determinants of poliovirus. This EV71 (S1-3′) strain is characterized by attenuated neurovirulence and limited spread of virus. In a subsequent study by Arita et al.,[29] three cynomolgus monkeys were inoculated with EV71 (S1-3′), followed by a lethal challenge with the parental virulent strain EV71 (BrCr-TR); they suffered mild neurological symptoms (tremor), but survived the lethal challenge without exacerbation of the symptoms. Moreover, the sera from the immunized monkeys showed a broad spectrum of neutralizing activities against different genotypes of EV71. These findings indicate that EV71 (S1-3′) acts as an effective antigen. However, it does cause mild neurological symptoms when inoculated via the intravenous route. Additional studies are required to ensure that further attenuation produces effective attenuated vaccine strains. EV71 infection via the oral route did not efficiently cause neurological disorders in the inoculated monkeys. Therefore the cynomolgus monkeys were inoculated by intravenous route instead of the oral route to evaluate the antigenicity of the attenuated EV71 vaccine. Thus, to develop an oral EV71 attenuated vaccine, a valid animal model of EV71 infection by the oral route is urgently needed.

Meanwhile, it is well known that in a small number of OPV recipients and their close contacts, especially those with primary humoral immunodeficiencies, the vaccine strain can mutate to a neurovirulent strain during OPV replication and cause vaccine-associated paralytic poliomyelitis (VAPP), which is an adverse side effect of OPV.[30–32] A 1969 World Health Organization Collaborative study found that the VAPP rate was one in every 5.9 million doses administered for vaccine recipients and one in every 6.7 million doses administered for contacts.[33] Therefore, the genetic stability of the attenuated OPV is a major concern, and efforts should be made to further attenuate the neurotoxic effects and increase the genetic stability of the attenuated EV71 vaccine before clinical use.

4. Subunit vaccine

To overcome the potential problem of reversion to virulence of attenuated strain vaccine, subunit vaccines consisting of only one or a few 'subunit' proteins of the pathogen that can stimulate immune responses directed at the intact virus have been developed using recombinant DNA technology. In common with other enteroviruses, the VP1, VP2, and VP3 of EV71 are responsible for the antigenic diversity of enteroviruses, but the VP1, the major capsid protein of EV71, is clustered with neutralization epitopes and has the potential to act as an antiviral subunit vaccine.[34]

Wu et al. [21] have described a recombinant VP1 protein expressed in *Escherichia coli* BL21, showing that the VP1 protein with a complete adjuvant is able to elicit a neutralizing antibody response, enhance T helper cell proliferation, and induce high levels of interleukin (IL)-10 and interferon (IFN)-γ in mice, providing direct evidence that the VP1 protein contains neutralizing epitopes independent of other viral capsid proteins; this paves the way for the use of VP1 as a backbone antigen for developing subunit vaccines against EV71.

Transgenic edible plants and mammalian glands are possible alternatives to prokaryotic and eukaryotic cell culture systems, offering a palatable oral delivery system, which can elicit a good mucosal immune response as well as systemic humoral and cellular immune responses, making it particularly suitable for protecting against infectious agents intruding via the mucosal surface.[35,36] EV71 initiates disease following implantation in the gut mucosa, showing the potential of an oral vaccine for immunization against EV71 infection. Chen and colleagues[37] have developed VP1-expressing transgenic tomato fruits. These were used as a mouse free-feeding oral vaccine. The VP1-specific fecal IgA and serum IgG were then observed in mice, and both humoral and cellular immunity against EV71 were established, showing the potential use of the transgenic tomato as an oral vaccine. Meanwhile, other EV71 oral VP1 vaccine delivery systems, such as milk of transgenic mice described by Chen et al.[38] and the Salmonella-based method by Chiu et al.[39] have been extensively explored.

For oral vaccines, gastric acid and enzymatic digestion are major concerns, since they may interfere with vaccine absorption. The *Enterovirus* genus can withstand human gastric acid and remain infectious below pH 3.0. However, VP1 is a capsid protein on the surface of the EV71 particle, and whether it can resist

human gastric acid has not been fully addressed. Meanwhile, digestive enzymes may also degrade the antigens. In the experiment of Chen et al., mice gavaged with VP1 protein produced more VP1-specific antibodies than mice fed transgenic tomato containing more VP1 protein, in both sera and feces, indicating that chewing and digestion cause degradation of VP1 antigen.[37] Also, the oral rotavirus VP4 protein vaccine has been shown to provide lower levels of antibodies and less protection than immunization by injection of the rotavirus VP4 protein,[40] indicating the possible interference of digestive enzymes. Moreover, it has been difficult to determine the precise dose of antigens for immunization, since competition with food and microbial antigens interferes with the absorption rate of vaccine components. Therefore, to improve oral vaccine delivery, many strategies have been developed, such as using tissue-specific promoters, the addition of mucosal immune adjuvant, using liposomes to protect the fusion peptides in the phospholipid bilayer vesicles from the gastric enzymes, and *N*-trimethyl chitosan nanoparticles.[41,42] For the oral EV71 VP1 subunit vaccine, exploring strategies to protect antigens from enzyme degradation in the gut is necessary.

5. DNA vaccine

DNA vaccines are expressed intracellularly in the same manner as during natural viral infection and can stimulate either humoral immunity, cellular immunity, or both.[43] Also, DNA vaccines only deliver the target subunit antigen and thus cause fewer adverse effects, making them another valuable vaccine choice for most viral infections.

Tung and co-workers[44] developed an EV71 DNA vaccine by inserting the VP1 gene into a eukaryotic expression vector and evaluated the immune response in mice. Their study results showed that the anti-VP1 IgG level increased in mice immunized with DNA vaccine; in contrast, this level declined after boosting immunization. Furthermore, although the anti-VP1 IgG exhibited neutralizing activity against EV71, the neutralizing effect of the sera of mice immunized with the VP1 DNA vaccine was much lower than that of EV71-infected human serum. Another DNA vaccine developed by Wu et al.,[21] elicited a high neutralization titer and stable titer level, which could be detected even at a late post-immunization time. However, because the DNA vaccine contains fewer antigenic epitopes, it induces a weaker immune stimulation than the whole virus particles. Therefore, strategies to increase the immune stimulation ability of DNA vaccines have been developed including: incorporation of immunostimulatory sequences in the backbone of the plasmid, co-expression of stimulatory molecules, use of localization/secretory signals, and an appropriate delivery system, as well as adjuvants and optimization of transgene expression.[43,45–47] All these techniques can help to prepare a better EV71 DNA vaccine.

6. Epitope peptide vaccine

An epitope peptide vaccine consisting of a well-defined immunogenic epitope stimulates an effective and specific protective immune response while avoiding potential undesirable effects.

The host immune response developed upon any viral infection is primarily CD4+ T cell-dependent, including the induction of a cytotoxic cellular response and efficient antibody response. Thus, identification of CD4+ T cell epitopes and B cell epitopes is of great importance in the design of effective epitope peptide vaccines. Foo and colleagues[48–50] have published several studies aimed at identifying the T-cell and B-cell epitopes of EV71. In these studies, they identified three regions, 66–77, 145–159, and 247–261, spanning amino acids of the VP1 protein; they showed that these three regions could induce proliferation of CD4+ T cells, then producing abundant IL-2 and IFN-γ upon stimulation. Additionally, among the three peptides, amino acids 145–159 induced the strongest proliferative response and highest cytokine production. Furthermore, in order to identify the neutralizing linear epitopes, 95 overlapping synthetic peptides spanning the VP1 capsid protein of EV71 were used to immunize mice.[49] Peptides containing amino acids 163–177 and 208–222 of VP1 protein were capable of eliciting neutralizing antibodies against EV71, and the neutralizing antibodies elicited by the synthetic peptide 208–222 were able to confer good in vivo passive protection against homologous and heterologous EV71 strains in suckling Balb/c mice.[50] Moreover, the monoclonal antibody generated by immunizing mice with amino acids 208–222 of VP1 showed strong neutralizing activity against EV71 in an in vitro neutralization assay.[51] Therefore, the epitope peptide vaccine represents a promising candidate for EV71. The identification of more T-cell and B-cell epitopes of the VP1 protein, as well as a combination epitope peptide vaccine, should be considered in the search for a more effective epitope peptide vaccine.

7. Virus-like particle (VLP) vaccine

VLPs are empty particles composed of all major structural proteins, mimicking the organizations and conformations of the native particles. To date, it has been shown that a wide range of VLPs of clinically important viruses (e.g., HIV and severe acute respiratory syndrome coronavirus) induce effective neutralizing antibodies and cytotoxic T cell responses.[52,53] The VLP vaccine, not only conserving the conformational epitopes, but also having no risk of virulent revertants, is also a promising vaccine strategy for EV71.

Hu et al.[16] and Chung et al.[54] used a recombinant baculovirus expression system to express the 3CD and P1 proteins of EV71; the 3CD protein cleaves P1 precursor into VP1, VP3, and VP0, which spontaneously assemble to form VLPs, inducing both Th1 and Th2 immune responses. More importantly, the VLP immunization of mother mice conferred protection to neonatal mice against the lethal viral challenge, indicating EV71 VLP to be a promising vaccine. Chung et al.[55] also found that compared with the intact VLPs, the denatured VLPs elicited significantly lower levels of neutralizing antibodies and conferred lower degrees of protection against virus challenge, which highlights the importance of preserving the conformation-dependent epitopes in preventing EV71 infection.

At present, the VLPs are mostly developed using insect cells and the strict culture conditions limit the required large scale of vaccine production. Thus, transgenic plants or yeast that can produce VLPs to be delivered by either oral administration or injection, might be promising expression systems.

8. Prospects

EV71 is one of the causative pathogens of HFMD, often complicated with neurological disorders. Due to the similarities between poliovirus and EV71 in many virological and clinical aspects, the success of the oral polio vaccine and inactivated-virus preparation in controlling poliomyelitis and eradicating the poliovirus, highlight the potential for controlling EV71 by vaccination. Poliovirus vaccine technology, both live attenuated and inactivated virus vaccines, can be adapted to control EV71 infection.

In recent years, various types of vaccine against EV71 have been developed, but these have as yet remained at the preclinical stage. Outbreaks of EV71 have been reported around the world since 1969. In economically developed nations, it typically causes a mild illness, and most patients usually recover quickly. However, since

the late 1990 s, there has been a significant increase in EV71 epidemics, and it has emerged as a serious threat to public health throughout the Asia-Pacific region. Developed countries with the resources for vaccine research and development do not view EV71 as a priority, and the vaccine industry in developed countries has little incentive to develop a vaccine to EV71. At present, there are only a few vaccine industries in the Asia-Pacific region undertaking EV71 vaccine preparation. Therefore, to effectively control EV71, more effort and cooperation worldwide is needed.

Because EV71 mainly threatens the children in developing countries, an ideal EV71 vaccine would have to be inexpensive, safe, convenient to administer, and acceptable to parents. For the inactivated virus vaccine, the established regulatory standards may allow a license to be obtained to meet the immediate demands for EV71 control. Due to the need to conserve the three-dimensional structure, the formalin-inactivated virus vaccine is a potential candidate vaccine for EV71. An oral EV71 attenuated vaccine has the potential to control EV71 in the same way as OPV controlling poliovirus, though further attenuation procedures are needed. At present, there are five genotypes of EV71. Cross-protection to the different genotypes for all the EV71 vaccines under current development is unclear. Hence, the preparation of a vaccine strain providing wide cross-protection is another important issue for EV71 vaccine development.

Conflicts of interest

There are no conflicts of interest with regard to employment, consultancy, stock ownership, honoraria, paid expert testimony, patent applications/registrations, or grants.

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