Parenteral, non-live rotavirus vaccine: recent history and future perspective

Since the widespread introduction of oral and live attenuated rotavirus vaccines around the world in 2009, the impacts of disease burden and the effects of disease reduction in developing countries have been proven. However, in low and middle-income countries, the vaccine efficacy is somewhat lower than in developed countries due to differences in nutritional conditions, microbial environments of individuals, and other factors. In addition, as oral, live vaccines have been found to be associated with rare but serious side effects, the development of a next-generation vaccine with safety, improved effectiveness, and ease of storage is currently underway. New vaccine strain developed by the Centers for Disease Control and Prevention in the United States are undergoing preclinical testing of efficacy, antigen dose, and administration route in the form of a heat-treated inactive vaccine, and a recombinant protein-based trivalent subunit vaccine developed by the Program for Appropriate Technology in Health is undergoing clinical trial in phase III. Several research groups are also developing non-replicating protein-based rotavirus vaccines using virus-like particles and nanoparticles. This review provides a brief overview of the development status and technology of parenteral, non-live rotavirus vaccines worldwide.

Keywords: Rotavirus, Vaccines, Parenteral, Non-live, Virus-like particles, Subunit

Introduction

Rotavirus (RV) is classified into seven groups (A to H), and viruses that infect humans belong to groups A and H. Group A RV is a major cause of acute gastroenteritis in children under 5 years of age, resulting in about half a million deaths annually before the vaccination program is implemented [1]. RotaShield was granted the first RV vaccine in 1998, but it was voluntarily withdrawn from the market in 1999 as it was proven to be related to intussusception after vaccination [1]. It took more than a decade for the second-generation vaccine, Rotarix and RotaTeq, to be used worldwide. Rotarix is a monovalent, live attenuated human RV vaccine that prevents the infection of G1 and non-G1 types of RV when administered as a two-dose series in infants [1]. In addition, some countries have been granted additional oral RV vaccines (RotaVac, RotavimM1, ROTASII1, and Lanzhou Ram RV vaccines) by the National Immunization Program. From 2005 to 2015, there was a significant decrease in the mortality rate from acute bowel disease in children under the age of 5 years, probably due to the effect of the introduction of the vaccine [2]. However, Rotarix and RotaTeq, currently used in more
than 80 countries’ vaccination programs, have significantly reduced the incidence of severe diarrhea by more than 80% in developed countries, while in developing counties only have a 50% effect [2]. This may be due to a combination of factors involved in the immune response of infants, including maternal antibodies, chronic enteropathy, microbiome, and interference from other infections, like other live attenuated oral vaccines such as oral polio, cholera, and typhoid. Costs and safety are also other concerns raised regarding the live oral RV vaccine. Indeed, the risk of RV vaccine associated illness is highlighted in severe immunodeficiency infants. In addition, oral RV vaccines provide protection against disease, but the risk of reassortment between the vaccine and wild type strain exists, requiring high manufacturing costs and a cold chain, requiring the development of alternative and efficient RV vaccines. As an alternative to this need, a new paradigm for the development of parenteral, non-live RV vaccines has been considered. This review investigated the development situation and potential of the new generation protein-based RV vaccine candidates utilizing VP6 and VP8 proteins as key antigens and aimed to provide an updated overview of their potential as vaccine candidates.

### Chemically and Physically Inactivated Rotavirus Vaccine

Formaldehyde, beta-propiolactone, and binary ethylenimine (BEI) have been commonly used to inactivate viruses such as influenza, polio, and Japanese encephalitis. Conner et al. [3] in 1993 showed that rabbits immunized with formalin-inactivated simian RV vaccines (strain SA11) via intramuscular route, were completely protected against rabbit RV infection.

In case of human RV, Yuan et al. [4], developed BEI-treating inactivated rotavirus vaccine (IRV) with attenuated Wa strain and evaluated vaccine efficacy using gnotobiotic pig. Gnotobiotic pigs are susceptible to both human and pig RV and develop diarrhea for at least 6 weeks after viral infection [5,6]. Intramuscular injection of gnotobiotic pig with BEI-IRVs showed significant increases in antigen specific antibody-secreting cells compared to non-immunized groups, but unfortunately did not show a significant protective immune response against viral challenge [5].

As has been observed in many other pathogens, inactivation of RV using chemical reagents has been shown to cause damage to the integrity of antigen in virus particles and reduced biological activity and neutralizing antibody production [7]. Unlike chemical treatments that cause structural changes in viral antigens, physical treatment, such as heat, ultraviolet (UV) and gamma-irradiation of viruses are commonly more desirable to maintain antigen integrity [8,9]. McNeal et al. [8] suggested that the RV inactivation methods using psoralen treatment and long-wavelength (365 nm, 40 minutes) UV light irradiation. Triple or double layered murine RV were inactivated by psoralen/UV and then immunized with mice with or without QS-21 adjuvant. Intramuscular immunization of psoralen/UV-IRV consistently induced RV specific immunoglobulin G (IgG) responses; however, protection was incomplete, and the virus shedding occurred [8]. Another study showed that the inactivation of RV using gamma-irradiation has been proposed as a new developing strategy for effective RV vaccines [9]. Shahrudin et al. [9] tested the immunogenicity of 50 kGy gamma-RV in mice corresponding amount of gamma rays that the virus could not replicate and showed the induction of RV-specific humoral immune responses comparable to those induced by live RV without using adjuvant. One thing to be solved in this research is that the inactivation curve of RV did not show logarithmic linear regression after exposure to increased gamma ray, so it was not possible to calculate the amount of radiation required to achieve internationally acceptable sterilization assurance level, which should be addressed later.

Recently, the most notable physical method for inactivation of RV is heat treatment. Jiang et al. [10] suggested a simple way to incubate the virus for 2 hours at 60°C to inactivate RV. Following this way, heat-treated YK-1 RV were morphologically similar to live virions and confirmed to be lack of infectivity. Mouse experiment was performed to determine the vaccine efficacy of the heat-IRV, and a two-dose immunization of heat-IRV without adjuvant resulted in high titers virus specific antibody in serum of mice.

The US Centers for Disease Control and Prevention (CDC) recently developed a candidate human vaccine strain CDC-9 that was isolated from fecal specimen of a child in the United States and has been shown to have good properties as a vaccine such as high yield in Vero cell culture and triple layered particle formation [11]. Jiang et al. [10] further demonstrated that heat-treated CDC-9 IRV was highly immunogenic and protected piglets from challenge when adjuvanted with alum and administered intramuscularly. CDC-9 is a single gene reassortant with the VP3 gene derived from a G2P4 virus and the others from a G1P8 virus [12]. Based on these serologic
properties, they also investigated heterotypic immunity of monovalent CDC-9 IRV. Sera from piglets that vaccinated with heat-treated CDC-9 IRV (G1P8) showed heterotypic neutralizing activity against non-G1P8 strains, including human MW-333 (G8P4) and human W79-bovine reassortant WC3 reassortant strain (G6P8). This finding suggested that heat treated human RV strain could provide broad cross-reactive immunity to different human genotypes [13]. The parenteral RV vaccines have comparative advantages over oral vaccines, but they are relatively disadvantageous for stimulating mucosal immunity, which is important in preventing RV. To overcome these limitations, Resch et al. [14] have attempted microneedle (MN) patch immunization, which is advantageous for activating mucosal immunity. In this study, it was confirmed that a similar or somewhat lower level of immune response was induced by coated MN of CDC-9 IRV, compared to intramuscular injection. However, the difference was not statistically significant, and there was also a positive effect such as dose sparing effect on MN vaccination [14].

Several combination vaccines are being used to relieve the burden of repeated vaccinations during infant. If IRV is added to the existing vaccine schedule in the future, the immunization schedule may overlap with polio vaccine; therefore, studies on the immune competition between IRV and other vaccines have been conducted. Wang et al. [15] tested in guinea pigs for formulations of IRV-IPV combination and rat model for IRV plus Salk IPV combination to determine whether co-administration might interfere with the immune response to each product. As a result, IRV administered alone or in combination with IPV did not impair the immune responses to either RV or poliovirus and similarly, IPV administered alone or together with IRV induced comparable levels of neutralizing antibody to poliovirus. These results demonstrate the potential use of IRV-IPV combined vaccines, and more specific studies will be needed in the future.

The last topic to be discussed in this section is not IRV itself, but cell technology for effective cultivation of RVs. Orr-Burks et al. [16] succeeded in discovering genes negatively involved in RV replication via siRNAs such as EMX2, WDR62, and LRGUK in Vero cell, and finally, EMX2 deleted cell line by CRISPR-Cas9 showed high yield of RV replication and antigen production compared to normal cell line. The development of an improved Vero vaccine cell line is expected to provide a solution that enables low cost and stable RV production.

**Truncated VP8 Subunit Vaccines**

One of the most advanced candidate for the parenteral RV is P2-VP8*P[8] based vaccine developed by Dr. Taka Hoshino which is a recombinant protein fused with truncated VP8* protein and P2 epitope derived from tetanus toxin which exerts a strong T cell responses [17]. RV infectivity requires proteolytic cleavage of the VP4 by host protease and the subsequent formation of VP5* and VP8* the distal portion of the VP4 spikes which interact with glycan receptors to facilitate viral attachment. Thus, the VP8* protein, which is essential for viral entry, can be a good candidate for vaccine antigen [18]. Based on this, the monovalent P2-VP8*P[8] consisted of VP8* subunit from the human RV Wa strain was produced in baculovirus or *Escherichia coli* expression system [19] and the immunogenicity and protection efficacy of P2-VP8* have been investigated in several animal models over the past decade [20-22]. Currently, P2-VP8* vaccine has entered clinical trials. The first clinical testing of the monovalent P2-VP8*P[8] was performed in 18–45-year-old adults in United States and demonstrated safety and immunogenicity of the vaccine [23]. These results led to aged descending and dose-escalating phase I clinical evaluation with toddlers and infants in South African. The monovalent P2-VP8*P[8] vaccine was generally well-tolerated and when local reactogenicity was reported, it was transient and never severe. Almost all vaccine recipients demonstrated robust IgG and immunoglobulin A (IgA) response to homologous RV after three vaccinations. Neutralizing antibody responses to heterologous RV strains were most robust to P[8] strains, moderate to the P[4] strain, and fairly limited to the P[6] strain. Based on these results, a trivalent vaccine that includes antigens from P[4], P[6], and P[8] strains has been developed to broaden responses for these three P-types. This clinical study (phase I/II double-blind, randomized, placebo-controlled, descending age, dose-escalation study of the safety, and immunogenicity of the trivalent P2-VP8 subunit RV vaccine in healthy South African adults, toddlers and infants, NCT02646891) is currently finished and the trivalent P2-VP8 RV vaccine was generally well tolerated at all dose levels tested in adults, toddlers, and infants [24]. Anti-P2VP8 IgG titers to P[4], P[6], and P[8] were high and similar for all three vaccine antigens. Almost 99%–100% infants across all vaccine groups had a sero-response 4 weeks after three vaccinations. Adjusted neutralizing antibody responses to each of the strains (P[4], P[6], and P[8]) were shown after the third
injection in 78%–81% of infants in the 30 μg and 90 μg dose groups, and were similar across all three strains. Neutralizing antibody responses to DS-1 (P[4]) and 1076 (P[6]) strains and IgG responses to P[4] and P[6] antigens were similar to those for the Wa (P[8]) strain.

Finally, a phase III clinical trial (A Phase 3 Double-blind, Randomized, Active Comparator-controlled, Group-sequential, Multinational Trial to Assess the Safety, Immunogenicity and Efficacy of a Trivalent Rotavirus P2-VP8 Subunit Vaccine in Prevention of Severe Rotavirus Gastroenteritis in Healthy Infants, NCT04010448) using the 90 μg dose of trivalent P2-VP8 subunit RV vaccine is underway to determine if it protects infants in Africa and Asia. PATH, also known as Program for Appropriate Technology in Health (Seattle, WA, USA), is the major support organization for this clinical trial, and SK biosciences (Seongnam, Korea) is making joint efforts to optimize the manufacturing process. The results are expected in late 2025.

Non-replicating Virus-Like Particles Vaccines

Non-enveloped RV particles are composed of triple-layered capsid, inner, middle, and outermost layer and contain 11 segments of double-stranded RNA as a viral genome. The RNA genome encodes six structural (VPs1–VP4, VP6, and VP7) and six non-structural proteins (NSP1–NSP6) [25]. The single-layer of viral capsid consist of VP2 protein and double-layered particles are composed with VP2 and VP6 proteins. VP7 trimers and protruding VP4 trimers form the outermost layer, resulting in triple-layered particles (Fig. 1) [25]. The virus-like particles (VLPs) self-assemble from viral capsid proteins, and they resemble native virus structurally and antigenically. Several attempts have been made to develop a vaccine using the double (dl) or triple (tl) layered VLP of the RV as a recombinant non-replicating rotavirus vaccine (NRRV). RV-dl or tl VLP approach is being developed by research group in Baylor College of Medicine. O’Neal et al. [26] have compared the immunogenicity and protective efficacy of (dl) 2/6-VLP and (tl) 2/6/7-VLPs vaccines with cholera toxin as an adjuvant administered by oral or intranasal routes in mice. Serum IgG and intestinal IgA responses were induced higher in intranasal group than oral group, and all mice receiving VLPs intranasally were protected from challenge without viral shedding. Similarly, Yuan et al. [27] generated recombinant (dl) 2/6-VLP derived from simian SA11 or human (VP6) Wa and bovine RF (VP2) RV strains using baculovirus expression system and investigated the vaccine efficacy in gnotobiotic pigs. Two to three vaccinations of the (dl) 2/6-VLP vaccine with LT-R192G, heat-labile toxin, to gnotobiotic pigs resulted in sufficient antigen-specific antibodies but failed to induce robust neutralizing [27]. As a results, (dl) 2/6-VLP administered intranasally with an adjuvant were immunogenic but did not confer protection against RV infection or diarrhea in gnotobiotic pigs. Therefore, while mucosal administrated (dl) or (tl) VLPs are promising for NRRV, but VLPs still need to be improved due to differences in effectiveness depending on anii-
nal models.

RV VP6 protein is composed of the middle layer of viral capsid and the most abundant structural protein in virion. Interestingly, VP6 does not induce neutralizing antibodies but it can confer protection against RV challenge and cross-protective immunity in murine model [28,29].

Although the immunological mechanism has not been completely defined, VP6 specific serum IgA level is thought to be a strong surrogate marker for vaccine induced protective immunity [30]. It has been found that self-assembly of VP6 protein alone resulted in structural polymorphisms such as nanotubes, spheres, and trimers depending on pH and other conditions [30].

The VP6 spherical particles formed at pH < 5.5 but they were heterogeneous in the size and diameter. On the other hand, in the pH range of 5.5-7.0, uniform large tube was formed, and in pH 7 and above, tubes with a small radius of 45 nm were assembled. Pastor et al. [31] showed that immunization of mice with VP6 nanotubes elicits an antibody response that correlates with protection against challenge with RV and confers a higher degree of protection than immunization with double-layered RV particles containing VP2 or VP6 trimers.

When the VP6 nanotubes were vaccinated with noroVLP or Coxsackie VLP (di or tri-valent) as a combination vaccine, robust protective immunities were induced against both rotavirus and Coxsackie, which also served as an immune modulating effect on VP6 nanotube [32-36]. On the other hand, recent studies have shown that VP6 nanotube does not exhibit adjuvant characteristics for monomeric antigens or short peptides [37]. Two types of small antigens, P particles derived from norovirus capsid protein and small peptides (23 mer) derived from extracellular matrix protein (M2e) of influenza virus were tested to demonstrate that VP6 nanotube had an immune supporting effect. Vaccination with P particles alone induced low level of IgG antibodies but the co-administration of VP6 nanotube with P particles showed significantly increased antibody responses. However, neither M2e peptide alone nor combination with VP6 nanotubes showed significant differences in serum IgG levels. This means that the immune modulating effect of VP6 nanotubes is only effective for antigens in particle form [37].

Another approach to develop non-replicating RV vaccines is to display antigen in nanoparticles. Nanoparticles are poly-peptide assemblies that present multiple copies of subunit in well-ordered arrays with defined orientations that potentially intimates the repeatability, size, and shape of the natural host-pathogen. Such nanoparticles provide multiple binding sites of antigens and can provide enhanced antigen stability and immunogenicity [38].

Cincinnati Children’s Hospital research group produced P24-VP8* nanoparticles in which the VP8* was inserted into the outermost surface loop of the P domain. Based on the previous studies, it was confirmed that a foreign antigen composed of more than 200 amino acids can be inserted into the loop region, and the antigen inserted into loop can induce effective immune responses [38]. The high immunogenicity and protective efficacy of the P24-VP8* nanoparticles were confirmed in mouse [21] and gnotobiotic pig model [39], and in some cases, these effects were observed even in the absence of adjuvant.

Xia et al. [40] in Cincinnati Children’s Hospital research group also investigated the usefulness of S60 nanoparticles as a vaccine platform. They introduced mutations including R69A and triple cysteine mutations (V57C/Q58C/S136C) in norovirus S domain for efficient assembly and produced icosahedral S60 nanoparticles using E. coli expression system. Then S60-VP8* chimeric particles displaying 60 RV VP8* proteins by fusion to the C-terminus of the S protein were generated and evaluated their vaccine potentials. Mice were then immunized with S60-VP8* nanoparticles and free VP8* protein as controls for comparison with alum adjuvant. After three dose vaccinations, significantly improved immunogenicity was observed in S60-VP8* nanoparticles group [40] and further experiments with S60-mVP8* nanoparticles in which murine VP8* antigens are expressed, showed protective immune responses against murine RV (epizootic diarrhea of infant mice) challenge in mice [41]. Overall, it has been shown that nanoparticles resulting from the properties of capsid proteins such as the S and P proteins of norovirus can be effectively utilized for the transfer of RV antigens to immune system [37].

In addition to viral nanoparticles, other naturally occurring self-assembling nanoparticles have been purpose. Bacterial ferritin, a protein whose main function is iron storage in cells, self-assembles into particle forms with robust thermal and chemical stability. Therefore, ferritin nanoparticles are potentially suitable for delivery antigens. Previous studies have shown that the Helicobacter pylori ferritin-based nanoparticles were generated in bacterial expression system to present a multivalent array of the influenza viral hemagglutinin protein with its native trimeric conformation and immunization of mice with these HA-ferritin nanoparticles showed improved
vaccine potency [42].

Similar study using Helicobacter pylori ferritin-based nanoparticles have been reported by Li et al. [43]. In this study, recombinant RV VP6 and ferritin fusion proteins were expressed in E. coli system and rVP6-ferritin nanoparticles were self-assembled to uniform spherical structure which similar to ferritin in vitro. Oral administration of rVP6-ferritin without the CTB (cholera toxin subunit B) adjuvant induced higher level of humoral immune responses than free rVP6 protein in mice. Moreover, the rVP6-ferritin nanoparticles were expressed in the milk of transgenic mice, and gavage of this milk induced a significant reduction in diarrhea symptoms during RV infection.

Collectively, these findings supported the use of nanoparticles or similar VLPs as efficient scaffolds for the presentation of heterologous RV antigens as vaccine platform.

Conclusion

Although two live attenuated RV vaccines are licensed in many countries, several concerns, such as safety issues, different efficacy between countries and high cost compared to efficacy are yet to be addressed. New approaches consisting of parenteral, non-replicating RV vaccines are currently undertaken which focused on inactivated CDC-9 RV strains, P2-VP8* recombinant protein based trivalent subunit and VP8* displayed VLPs and nanoparticles. Given the desirable characteristics of the recombinant protein vaccines are immunogenic with safety and the possibility of fast and convenient manufacturing, the parenteral, recombinant protein vaccines can be considered as an alternative non-live RV vaccine.

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