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Inclusion of a universal tetanus toxoid CD4⁺ T cell epitope P2 significantly enhanced the immunogenicity of recombinant rotavirus VP8* subunit parenteral vaccines

Xiaobo Wen^{a,1}, Ke Wen^b, Dianjun Cao^{a,b}, Guohua Li^b, Ronald W. Jones^a, Jianping Li^c, Shousun Szu^c, Yasutaka Hoshino^{a,*}, and Lijuan Yuan^{b,*}

^aRotavirus Vaccine Development Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America

^bDepartment of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, United States of America

^cLaboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

Currently available live oral rotavirus vaccines, Rotarix® and RotaTeq®, are highly efficacious in developed countries. However, the immunogenicity and efficacy of such vaccines in some developing countries are low. We reported previously that bacterially-expressed rotavirus VP8* subunit vaccine candidates with P[8], P[4] or P[6] specificity elicited high-titer virus neutralizing antibodies in animals immunized intramuscularly. Of note was the finding that antibodies induced with the P[8] VP8* vaccine neutralized both homotypic P[8] and heterotypic P[4] rotavirus strains to high titer. To further improve its vaccine potential, a tetanus toxoid universal CD4⁺ T cell epitope P2 was introduced into P[8] or P[6] VP8* construct. The resulting recombinant fusion proteins expressed in *Escherichia coli* were of high solubility and were produced with high yield. Two doses (10 or 20µg/dose) of the P2-P[8] VP8* vaccine or P2-P[6] VP8* vaccine with aluminum phosphate adjuvant elicited significantly higher geometric mean homologous neutralizing antibody titers than the vaccines without P2 in intramuscularly immunized guinea pigs. Interestingly, high levels of neutralizing antibody responses induced in guinea pigs with 3 doses of the P2-P[8] VP8* vaccine persisted for at least 6 months. Furthermore, in the

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*lyuan@vt.edu (LY); thoshino@niaid.nih.gov (YH).

¹Current address: College of Animal Science & Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang Province, China.

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gnotobiotic piglet challenge study, three intramuscular doses (50µg/dose) of the P2-P[8] VP8* vaccine with aluminum phosphate adjuvant significantly delayed the onset of diarrhea and significantly reduced the duration of diarrhea and the cumulative diarrhea score after oral challenge with virulent human rotavirus Wa (G1P[8]) strain. The P2-P[8] VP8* vaccine induced serum virus neutralizing antibody and VP4-specific IgG antibody production prechallenge, and primed the pigs for higher antibody and intestinal and systemic virus-specific IFN-γ producing CD4+ T cell responses postchallenge. These two subunit vaccines could be used at a minimum singly or preferably in bivalent formulation to provide antigenic coverage of most of the G types of global importance.

Keywords

Human rotavirus; Subunit vaccine; T cell epitope; Immunogenicity; Protective efficacy; Diarrhea; Gnotobiotic pigs

Introduction

Rotaviruses are the single most important cause of severe diarrheal disease in infants and young children worldwide, responsible for an estimated >500,000 deaths annually among children aged <5 years, predominantly in developing countries [1]. In 2009, the World Health Organization recommended that two live oral rotavirus vaccines, Rotarix® (GlaxoSmithKline) and RotaTeq® (Merck), be included in routine childhood vaccination programs in all countries [2]. These two vaccines have been shown to be safe and efficacious in both developed and developing countries [3]. However, the immunogenicity and efficacy of these vaccines in selected low-income countries of Africa, Asia and Central America where the vaccines are needed most are low [4]. In addition, there are other concerns associated with these vaccines including an association with a risk of intussusception [5]; contamination with infectious porcine circovirus (PCV) and/or PCV DNA [6-8]; a diarrheagenic potential in infants with severe combined immunodeficiency [9]; and a reassortant rotavirus formation between vaccine strains and circulating wild-type strains or among vaccine strains with a potentially increased virulence as reported recently [10]. PATH (an international nonprofit organization aiming to improve the health of people around the world) is currently investigating non-replicating rotavirus vaccine (NRRV) candidates that may eliminate the risk of intussusception completely [11]. NRRVs that would be administered via non-oral routes may overcome factors that can lessen an oral vaccine's impact including co-infections in the digestive system or the presence of maternally-derived antibodies. Furthermore, NRRVs could potentially be included in a pediatric combination vaccine to simplify delivery.

Rotavirus outer capsid spike protein VP4, in the presence of trypsin, is cleaved to produce VP8* (~28 kDa) and VP5* (~60 kDa). The VP8* protein generated in various expression systems has been reported to induce virus neutralizing antibodies (VNtAbs) and an associated protection in mice [12]. We recently constructed bacterially-expressed VP8* subunit vaccine candidates containing amino acid (aa) residues 64 (or 65)-223 with P[8], P[4] or P[6] specificity [13]. Such VP8* vaccines elicited high levels of homotypic and

varying levels of heterotypic VNtAbs in animals immunized intramuscularly (IM), thus demonstrating a solid vaccine potential.

In general, the magnitude of protection provided by a parenteral vaccine against orally-transmitted diseases varies depending upon the levels of protective antibodies induced by the vaccine [14]. Although our VP8* vaccines are reasonably immunogenic, we aimed to further increase the immunogenicity by incorporating a universal T cell epitope. Selected T cell epitopes from tetanus toxoid (TT) have been extensively studied. They are shown to be widely recognized in association with a large number of MHC class II molecules and to be universally immunogenic in humans and mice [15]. For example, the P2 universal CD4⁺ T cell epitope (aa 830-844) has been reported to enhance the immunogenicity of a synthetic peptide vaccine for malaria [16] as well as tandem copies of a rotavirus VP8 epitope [17]. In addition, the P2 has been proven to be as efficient as the whole TT molecule as a carrier without inducing epitope suppression [18]. We introduced the P2 into the P[8] or P[6] VP8* construct and expressed it in *Escherichia coli* (*E. coli*). We then characterized the immunogenicity and protective efficacy of these recombinant fusion proteins in guinea pigs and gnotobiotic (Gn) pigs, respectively.

2. Materials and methods

2.1. Viruses

Cell culture-adapted human rotavirus (HRV) Wa (G1P[8]) [19] and 1076 (G2P[6]) [20] strains were grown in primary African green monkey kidney cells [21]. The virulent Wa strain (the pooled intestinal contents from the 27th passaged Gn pigs) was used for challenge of Gn pigs at a dose of $\sim 10^5$ fluorescence forming units (FFU). The 50% infectious dose (ID₅₀) and 50% diarrhea dose (DD₅₀) of the virulent Wa in Gn pigs was determined as approximately 1 FFU [22]. The virus titer was determined by cell culture immunofluorescence (CCIF) assay and was expressed as FFU/ml as described previously [23].

2.2. Vaccine plasmid construction

Rotavirus gene cDNA of Wa or 1076 strain was obtained by a RT-PCR procedure as described previously [21]. The primers designed according to the genomic sequence of RNA segment 4 of each strain [GenBank accession numbers: FJ423116 (Wa) and M88480 (1076)] were as follows: 5'-TACTCATATGCAGTATATAAAAGCAAATTCTAAATTTATAGGTATAACTGAACTAGGCTCCGGCTCAGGC TTAGATGGTCCTTATCAGCC-3' (P2-P[8] VP8* sense), T cell epitope P2 is italicized, and the linker sequence encoding GSGSG is in bold. 5'-TAGAGCTCTATCACAGACCATTATTAATATATTCATTAC-3' (P2-P[8] VP8* antisense), *Nde* I and *Sac* I sites are underlined, and two stop codons are in bold. 5'-CGCGAACAGATTGGAGGTCAGTATATAAAAGCA AATTCTAAATTTATAG-3' (P2-P[6] VP8* sense), 5'-GTGGCGGCCGCTCTATTATAACCCAGTATTTATATATTCATTACACTTAG-3' (P2-P[6] VP8* antisense), flanking sequence identical to the vector is underlined, and a stop codon is in bold. P2-VP8* cDNA was synthesized as described previously [21]. The fusion

P2-P[8] VP8* fragment was amplified with cDNA as the template by an iProof High-Fidelity PCR system (Bio-Rad) and then cloned into pET28a vector (Novagen) harboring a 6×histidine tag, yielding pET28a-P2-P[8] VP8* plasmid. The P2-P[6] VP8* product was cloned into a linearized pETite vector (Lucigen) encoding a small ubiquitin-related modifier (SUMO) and 6×histidine tag at the N-terminus by homologous recombination without acquiring single strands according to manufacturer's instructions. The integrity and fidelity of amplification were confirmed by DNA sequencing.

2.3. Expression of recombinant proteins

The expression plasmid pET28a-P2-P[8] VP8* or pETite-P2-P[6] VP8* was transformed into competent *E. coli* BL21(DE3) pLysS cells or HI-control BL21(DE3) cells by heat shock. A single colony was inoculated into LB broth containing 50µg/ml kanamycin. When absorbance at 600nm reached 0.5, the expression of each fusion protein was induced as described previously [21]. Proteins were analyzed by Western Blot assay with a hyperimmune guinea pig antiserum (1:50) raised against Wa (P[8]) or ST3 (P[6]) strain as described [13].

2.4. Purification of P2-P[8] VP8* and P2-P[6] VP8* proteins

Each protein was purified by affinity chromatography as described previously [21]. The SUMO tag at N-terminus of P2-P[6] VP8* was removed using the SUMO express protease (Lucigen) according to manufacturer's instructions. The purity of recombinant proteins was confirmed by SDS-PAGE followed by a removal of imidazole in solution using a centrifugal filter unit (Millipore). The concentration of purified proteins and the level of endotoxin in each purified protein were quantified as previously described [21].

2.5. Immunization of guinea pigs

Outbred female Hartley guinea pigs weighing 500-550g (Charles River) were maintained under animal biosafety level 2 conditions in isolator cages. Guinea pigs were immunized IM three times at 2-week intervals with 10 or 20µg of the P[8] VP8* or P2-P[8] VP8*, or 20µg of P[6] VP8* or P2-P[6] VP8* vaccine with or without aluminum phosphate (AP) adjuvant (ADJU-PHOS®, Brenntag) (aluminum content of 100µg/dose). In a VNtAb kinetics study, guinea pigs were vaccinated IM with 10 or 20µg of P2-P[8] VP8* vaccine with AP three times at 2-week intervals. Blood samples were collected prior to each immunization as well as at 7 days or 2, 4 and 6 months after the third immunization. All guinea pig experiments were conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases.

2.6. Immunization and challenge of Gn pigs

Gnotobiotic pigs were derived by hysterectomy from near-term sows (Landrace and Large White crossbred) and maintained in germ-free isolator units [24]. Pigs were fed commercial ultra-high-temperature-treated sterile milk. All pigs were confirmed as seronegative for rotavirus and germ-free prior to immunization. The pigs were immunized IM three times (50µg/dose) with the P2-P[8] VP8* vaccine with AP (600µg/dose, in 0.5ml total volume) at

5 days of age (post-inoculation day [PID] 0) and on PID 10 and 21. At PID 28, the vaccinated (n=5) and mock-vaccinated control pigs (n=9) were challenged orally with 10^5 FFU of the virulent Wa to assess the protection against G1P[8] rotavirus-induced diarrhea and fecal virus shedding. Pigs were monitored and fecal swabs were collected daily from post-challenge day (PCD) 0 to 7 for detection of virus shedding. Fecal consistence scores were recorded to assess severity of diarrhea as described [25]. Rotavirus shedding was detected by an antigen ELISA (detect viral antigen) and virus titers were determined by CCIF (detect infectious virus particle) as described [25]. Blood samples were collected weekly before challenge and on PCD 2, 4 and 10. Pigs were euthanized on PCD 10 and mononuclear cells (MNC) from ileum, spleen and blood were isolated for detection of IFN- γ producing CD4⁺ and CD8⁺ T cell responses as described [26]. All Gn pig experiments were conducted in accordance with protocols approved by Institutional Animal Care and Use Committees of Virginia Polytechnic Institute and State University.

2.7. Neutralization, ELISA and immunocytochemical staining assays for detection of antibody responses

VNtAb titers in serum samples were determined by 60% plaque reduction neutralization assay [27]; titers of serum IgA antibodies to Wa HRV were determined by an ELISA [28]; and titers of serum IgA and IgG antibodies to homotypic VP4 were determined using an Sf-9 cell staining assay [29]. Samples negative at a dilution of 1:4 were assigned a titer of 1:2 for the calculation of geometric mean antibody titers.

2.8. Intracellular cytokine staining and flow cytometry analysis

Flow cytometry was used to determine frequencies of rotavirus-specific IFN- γ producing CD4⁺ and CD8⁺ T cells in ileum, spleen and blood of pigs as previously described [26]. Semi-purified Wa HRV antigen was used to stimulate MNCs in cell culture (12 μ g/ml) for 17 h. PHA (10 μ g/ml) and mock stimulated (medium-only) MNCs were included as positive and background controls, respectively. At least 100,000 cells were acquired on a FACSaria flow cytometer (BD Biosciences). Data were analyzed using FlowJo 7.2.2 software (Tree Star). The frequencies of IFN- γ ⁺ CD4⁺ and IFN- γ ⁺ CD8⁺ T cells were expressed as percentages among total CD3⁺ T cells. All mean frequencies were reported after subtraction of the background frequencies.

2.9. Statistical analysis

For comparison of geometric mean serum VNtAb titers (GMT) elicited in guinea pigs between different doses of P[8] VP8* and P2-P[8] VP8* vaccines and P[6] VP8* and P2-P[6] VP8* vaccines, mean duration of virus shedding and diarrhea, and mean cumulative fecal consistence scores between Gn pig groups, we used one-way analysis of variance (ANOVA)-general linear model (GLM) followed by Duncan's multiple range test. The HRV-specific IgA antibody titers, VP4-specific antibody titers, VNtAb titers, and virus shedding titers in Gn pigs between vaccine and control groups were compared using Kruskal-Wallis rank sum test. Proportions of virus shedding and diarrhea among pig groups were compared using Fisher's exact test. Statistical significance was assessed at $P < 0.05$, if

not specifically noted. All statistical analysis was performed using SAS program 9.2 (SAS Institute).

3. Results

3.1. Expression, purification and specificity of recombinant proteins

Both P2-P[8] VP8* and SUMO-P2-P[6] VP8* proteins were expressed in soluble and insoluble forms even at optimized parameters. The yield of purified P2-P[8] VP8* reached ~46mg/L culture, whereas that of purified P2-P[6] VP8* was ~10mg/L. This difference in yield was in part due to the different expression systems used; the former used a pET expression system and the latter employed a SUMO expression system which required an additional purification step—a cleavage and removal of the SUMO tag off the fusion protein. The expression and specificity of each purified protein was confirmed by SDS-PAGE (Figs. 1A and 1C) and Western blot analyses (Figs. 1B and 1D). The endotoxin levels were 1.837 EU/ml for P2-P[8] VP8* and 1.842 EU/ml for P2-P[6] VP8*, which were far less than recommended endotoxin levels (<20 EU/ml) for recombinant subunit vaccines [30].

3.2. Inclusion of P2 epitope enhanced the immunogenicity of VP8* proteins

VNtAb responses induced by the P2-P[8] VP8* or P[6] VP8* with AP adjuvant in guinea pigs after IM immunization are shown in Fig. 2. Because the VNtAb titers did not differ significantly between the 10µg and 20µg vaccine dose groups, the data were combined. After one dose of either vaccine, none of the animals developed a detectable VNtAb response against the homologous WaP[8] strain, from which the vaccine was derived. After 2 doses of the P2-P[8] VP8*, however, animals developed robust VNtAb responses with a GMT of 1:3620 (Fig. 2A). In contrast, the P[8] VP8* induced a significantly lower GMT of 1:640. Similarly, 2 doses of the P2-P[6] VP8* induced high levels of VNtAbs to the homologous 1076P[6] strain (GMT 1:905), whereas 2 doses of the P[6] VP8* induced significantly lower levels of antibodies (GMT 1:80) (Fig. 2B). Three doses of the P2-P[8] VP8* induced significantly higher levels of antibodies (GMT 1:10,240) than the P[8] VP8* (GMT 1:7,241). Similarly, 3 doses of the P2-P[6] VP8* or P[6] VP8* with AP induced high levels of homologous VNtAbs (57,926); however, no differences in GMTs were detected between the two vaccines.

After 3 doses of the P2-P[8] VP8*, guinea pigs developed high levels of homotypic VNtAbs against P[8] strains with G1, G3, G4 or G9 specificity (GMT 1:3,948) (Table 1). Furthermore, this vaccine also induced high levels of heterotypic VNtAbs against P[4] strains with G2, G8 or G12 specificity (GMT 1:1,522). Levels of heterotypic VNtAbs to P[6] and P[10] strains were low.

3.3. Addition of AP adjuvant enhanced the immunogenicity of P2- VP8* vaccines

The levels of VNtAbs induced by IM immunization with P2- VP8*, with or without AP, in guinea pigs are shown in Figs. 3A and 3B. Animals that received 2 doses of the P2-P[8] VP8* without AP developed moderate to high levels of homologous antibodies (GMT 1:761). The addition of AP elicited significantly higher (4-fold) VNtAb titers (GMT 1:3,044) after 2 doses (Fig. 3A). Similarly, homologous VNtAb titers induced by 2 doses of

the P2-P[6] VP8* with AP were significantly higher (GMT 1:905) than those without AP (GMT 1:106) (Fig. 3B). After 3 doses of the P2-P[8] VP8*, with or without AP, animals developed high levels of VNtAbs; however, the presence of AP elicited 2 times higher titers (without AP: GMT 1:5,120; with AP, GMT 1:10,240). Animals immunized three times with the P2-P[6] VP8*, with or without AP, developed high levels of homologous VNtAbs.

3.4. The kinetics of VNtAb response evoked by P2-P[8] VP8* vaccine

Guinea pigs immunized IM with 10 or 20µg/dose of the vaccine with AP developed high levels of VNtAb after a second dose as expected (Fig. 3C). A GMT reached 1:10,240 within two months after the third dose. This high level of antibody response was maintained for 6 months, when the study was terminated (Fig. 3C).

3.5. P2-P[8] VP8* vaccine induced high titers of serum VNtAbs and VP4-specific IgG antibody response, and primed the pigs for IgA antibody response postchallenge

Significantly higher titers of VNtAb titers were detected in serum of vaccinated pigs at challenge (PID 27) and postchallenge (PCD 2 and 10) compared to the control pigs ($P < 0.0001$) (Fig. 4A). HRV-specific IgA antibody was not detectable prechallenge (Fig. 4B). However, significantly higher IgA antibody titers at PCD 10 in the vaccinated pigs compared to the control pigs suggest that the P2-P[8] VP8* IM vaccine primed the pigs for serum IgA antibody production postchallenge. The P[8]VP4-specific IgG antibody titers increased significantly after each vaccination and after challenge (Fig. 4C). However, VP4-specific IgA antibody responses in serum were not detected pre- or postchallenge, which is consistent with the lack of detectable serum virus-specific IgA antibodies until PCD 10. Because IM immunization does not induce mucosal antibody responses [31], intestinal IgA and IgG were not measured in this study.

3.6. P2-P[8] VP8* vaccine induced rotavirus-specific IFN- γ producing T cell responses in intestinal and systemic lymphoid tissues

Pigs vaccinated IM with P2-P[8] VP8* and orally challenged with the virulent rotavirus had significantly higher frequencies of virus-specific IFN- γ producing CD8⁺ T cells in ileum compared to the challenged control pigs at PCD 10. Vaccinated pigs also had significantly higher frequencies of IFN- γ producing CD4⁺ T cells in ileum, spleen and blood (Fig. 4D), suggesting that IM immunization with the P2-P[8] VP8* effectively induced not only systemic but also intestinal virus-specific memory T cell responses.

3.7. P2-P[8] VP8* vaccine conferred protection against rotavirus diarrhea in Gn pigs

After oral challenge with the virulent Wa (G1P[8]), the pigs vaccinated IM with 3 doses of the P2-P[8] VP8* had significantly delayed onset of diarrhea (mean onset days from 1.4 to 4.2) and significantly reduced duration of diarrhea (mean duration from 5.6 to 1.6 days) compared to the controls (Fig. 5A), although all vaccinated pigs had diarrhea for 1-3 days. Their diarrhea scores were also significantly reduced compared to the controls (from 14.4 to 8.8) (Fig. 5B). All vaccinated pigs and control pigs shed virus and the amount of infectious virus shed in feces of the vaccinated pigs was similar compared to the controls (Fig. 5C).

4. Discussion

In this study, we inserted a universal CD4⁺ T cell epitope P2 from TT into recombinant subunit VP8* protein with P[8] or P[6] specificity and demonstrated that the resulting fusion proteins (i.e., P2-P[8] VP8* and P2-P[6] VP8*) with AP adjuvant elicited in guinea pigs significantly higher levels of homotypic VNtAbs after a second dose than the VP8* vaccines without P2. Moreover, in Gn piglets, three doses of the P2-P[8] VP8* significantly delayed the onset of diarrhea and reduced the duration of diarrhea as well as diarrhea cumulative scores after oral challenge with virulent P[8]HRV. The neonatal Gn pig model of HRV infection and diarrhea is a well-established model for the study of immunogenicity and protective efficacy of candidate HRV vaccines [32]. Virulent Wa strain (G1P1A[8]) HRV causes diarrhea and pathological changes in Gn pigs similar to those of human infants [22]. Various HRV vaccine candidates including live attenuated, inactivated, virus-like particle and DNA vaccines have been evaluated in the Gn pig model in our previous studies [25, 26, 31, 33, 34]. The magnitude of intestinal IFN- γ producing CD8⁺ T cells has been shown to positively correlate with the protection against rotavirus diarrhea [26]. Despite the small sample size in this Gn pig study, the results are encouraging and further studies in Gn pigs are needed to draw a conclusion for the efficacy of the P2- VP8* vaccines against diarrhea and the lack of protection against virus shedding. Nonetheless, we have shown that the P2- VP8* proteins may represent promising NRRVs. Importantly, such vaccines are free from various concerns associated with live oral rotavirus vaccines and may work better in low-income regions of the world where the vaccine is needed most. Of note, evidence from licensed vaccines has proven that parenteral vaccines are successful in preventing orally transmitted diseases including poliovirus, hepatitis A, *Vibrio cholera* and *Salmonella typhi* [35].

Tetanus toxoid (TT) has traditionally been considered to be a good carrier molecule to develop new vaccines for poorly immunogenic protective epitopes (such as small peptides) because of its widespread use in vaccination of humans without causing any serious harmful side effects. Individuals previously vaccinated with TT, however, fail to mount an effective immune response to a synthetic peptide injected as a conjugate with TT, although their capacity to make antibodies to TT itself remains uncompromised. This phenomenon of epitope-specific suppression [36], observed with other carrier proteins (e.g., egg lysozyme [37]), has been attributed in part to the presence of the carrier-specific suppressor T and B cells in the hosts previously administered with the carrier protein alone [38]. In comparison with TT, universal T cell epitopes have been demonstrated to work in a way as a carrier protein as well as an immunostimulator without inducing epitope-specific suppression [18]. For example, the P2 improved the immunogenicity of synthetic malaria peptide vaccines [16]. This immunity enhancing effect of a TT universal T cell epitope is thought to be due to an activation of specific T cells for synthesis of certain cytokines that are necessary for T and B cell activation and interaction as well as proliferation [39].

Extensive global epidemiological surveys have established that G1-G4 as well as P[8] and P[4] are of global epidemiologic importance [40]. Of note, unusual G and P types causing a high incidence of human infection have been reported recently in various regions of the world. These include G5, G8, G9, G10, G12, and P[6] [41]. Since, in nature, almost all HRV

G types have been detected in combination with P[8], P[4] or P[6] specificity, the P2-P[8] VP8* and P2-P[6] VP8* vaccines generated in this study could be used at a minimum singly or preferably in bivalent formulation to provide antigenic coverage to all the rotavirus G (VP7) types that are associated with P[8] or P[6] VP8 types of global, as well as regional, epidemiologic importance including new and emerging G8, G9 and G12 strains.

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References

- [1]. Tate, JE.; Burton, AH.; Boschi-Pinto, C.; Steele, AD.; Duque, J.; Parashar, UD. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. 20120127(1474-4457 (Electronic))
- [2]. Meeting of the immunization Strategic Advisory Group of Experts. April 2009--conclusions and recommendations. Wkly Epidemiol Rec. Jun 5; 2009 84(23):220–36.
- [3]. McCormack PL, Keam SJ. Rotavirus vaccine RIX4414 (Rotarix): a review of its use in the prevention of rotavirus gastroenteritis. Paediatr Drugs. 2009; 11(1):75–88. [PubMed: 19127963]
- [4]. Madhi SA, Cunliffe NA, Steele D, Witte D, Kirsten M, Louw C, et al. Effect of human rotavirus vaccine on severe diarrhea in African infants. N Engl J Med. Jan 28; 2010 362(4):289–98. [PubMed: 20107214]
- [5]. Rotavirus vaccine and intussusception: report from an expert consultation. Wkly Epidemiol Rec. Jul 22; 2011 86(30):317–21.
- [6]. Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, et al. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol. Jun; 2010 84(12):6033–40. [PubMed: 20375174]
- [7]. McClenahan SD, Krause PR, Uhlenhaut C. Molecular and infectivity studies of porcine circovirus in vaccines. Vaccine. Jun 24; 2011 29(29-30):4745–53. [PubMed: 21569811]
- [8]. Beach NM, Cordoba L, Kenney SP, Meng XJ. Productive infection of human hepatocellular carcinoma cells by porcine circovirus type 1. Vaccine. Oct 6; 2011 29(43):7303–6. [PubMed: 21742002]
- [9]. Addition of severe combined immunodeficiency as a contraindication for administration of rotavirus vaccine. MMWR Morb Mortal Wkly Rep. Jun 11; 2010 59(22):687–8. [PubMed: 20535093]
- [10]. Payne DC, Edwards KM, Bowen MD, Keckley E, Peters J, Esona MD, et al. Sibling Transmission of Vaccine-Derived Rotavirus (RotaTeq) Associated With Rotavirus Gastroenteritis. Pediatrics. Jan 25.2010 125:438–41.
- [11]. PATH. Vaccines for the future. News from PATH's Vaccine Development Program. Apr.2012 : 2–3.
- [12]. Dunn SJ, Fiore L, Werner RL, Cross TL, Broome RL, Ruggeri FM, et al. Immunogenicity, antigenicity, and protection efficacy of baculovirus expressed VP4 trypsin cleavage products, VP5(1)* and VP8* from rhesus rotavirus. Arch Virol. 1995; 140(11):1969–78. [PubMed: 7503695]
- [13]. Wen X, Cao D, Jones RW, Li J, Szu S, Hoshino Y. Construction and Characterization of Human Rotavirus Recombinant VP8* Subunit Parenteral Vaccine Candidates. Vaccine. 2012

- [14]. Offit PA, Clark HF. Maternal antibody-mediated protection against gastroenteritis due to rotavirus in newborn mice is dependent on both serotype and titer of antibody. *J Infect Dis.* Dec; 1985 152(6):1152–8. [PubMed: 2999254]
- [15]. Kaumaya PT, Kobs-Conrad S, Seo YH, Lee H, VanBuskirk AM, Feng N, et al. Peptide vaccines incorporating a 'promiscuous' T-cell epitope bypass certain haplotype restricted immune responses and provide broad spectrum immunogenicity. *J Mol Recognit.* Jun; 1993 6(2):81–94. [PubMed: 7508238]
- [16]. Franke ED, Corradin G, Hoffman SL. Induction of protective CTL responses against the *Plasmodium yoelii* circumsporozoite protein by immunization with peptides. *J Immunol.* Oct 1; 1997 159(7):3424–33. [PubMed: 9317141]
- [17]. Kovacs-Nolan J, Mine Y. Tandem copies of a human rotavirus VP8 epitope can induce specific neutralizing antibodies in BALB/c mice. *Biochim Biophys Acta.* Dec; 2006 1760(12):1884–93. [PubMed: 16978788]
- [18]. Kumar A, Arora R, Kaur P, Chauhan VS, Sharma P. "Universal" T helper cell determinants enhance immunogenicity of a *Plasmodium falciparum* merozoite surface antigen peptide. *J Immunol.* Mar 1; 1992 148(5):1499–505. [PubMed: 1371529]
- [19]. Wyatt RG, James WD, Bohl EH, Theil KW, Saif LJ, Kalica AR, et al. Human rotavirus type 2: cultivation in vitro. *Science.* Jan 11; 1980 207(4427):189–91. [PubMed: 6243190]
- [20]. Hoshino Y, Wyatt RG, Flores J, Midthun K, Kapikian AZ. Serotypic characterization of rotaviruses derived from asymptomatic human neonatal infections. *J Clin Microbiol.* Mar; 1985 21(3):425–30. [PubMed: 2984247]
- [21]. Wen X, Cao D, Jones RW, Li J, Szu S, Hoshino Y. Construction and characterization of human rotavirus recombinant VP8* subunit parenteral vaccine candidates. *Vaccine.* Sep 21; 2012 30(43):6121–6. [PubMed: 22885016]
- [22]. Ward LA, Rosen BI, Yuan L, Saif LJ. Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs. *J Gen Virol.* Jul; 1996 77(Pt 7):1431–41. [PubMed: 8757984]
- [23]. Liu F, Li G, Wen K, Bui T, Cao D, Zhang Y, et al. Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics. *Viral Immunol.* Apr; 2010 23(2):135–49. [PubMed: 20373994]
- [24]. Meyer RC, Bohl EH, Kohler EM. Procurement and Maintenance of Germ-Free Seine for Microbiological Investigations. *Applied microbiology.* Jul.1964 12:295–300. [PubMed: 14199016]
- [25]. Yuan L, Ward LA, Rosen BI, To TL, Saif LJ. Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. *J Virol.* May; 1996 70(5):3075–83. [PubMed: 8627786]
- [26]. Yuan L, Wen K, Azevedo MS, Gonzalez AM, Zhang W, Saif LJ. Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs. *Vaccine.* Jun 19; 2008 26(26):3322–31. [PubMed: 18456375]
- [27]. Hoshino Y, Jones RW, Kapikian AZ. Serotypic characterization of outer capsid spike protein VP4 of vervet monkey rotavirus SA11 strain. *Arch Virol.* 1998; 143(6):1233–44. [PubMed: 9687880]
- [28]. Parreno V, Hodgins DC, de Arriba L, Kang SY, Yuan L, Ward LA, et al. Serum and intestinal isotype antibody responses to Wa human rotavirus in gnotobiotic pigs are modulated by maternal antibodies. *J Gen Virol.* Jun; 1999 80(Pt 6):1417–28. [PubMed: 10374959]
- [29]. Yuan L, Honma S, Ishida S, Yan XY, Kapikian AZ, Hoshino Y. Species-specific but not genotype-specific primary and secondary isotype-specific NSP4 antibody responses in gnotobiotic calves and piglets infected with homologous host bovine (NSP4[A]) or porcine (NSP4[B]) rotavirus. *Virology.* Dec 5; 2004 330(1):92–104. [PubMed: 15527837]
- [30]. Brito LA, Singh M. Acceptable levels of endotoxin in vaccine formulations during preclinical research. *J Pharm Sci.* Jan; 2011 100(1):34–7. [PubMed: 20575063]

- [31]. Yuan L, Kang SY, Ward LA, To TL, Saif LJ. Antibody-secreting cell responses and protective immunity assessed in gnotobiotic pigs inoculated orally or intramuscularly with inactivated human rotavirus. *J Virol.* Jan; 1998 72(1):330–8. [PubMed: 9420231]
- [32]. Yuan L, Saif LJ. Induction of mucosal immune responses and protection against enteric viruses: rotavirus infection of gnotobiotic pigs as a model. *Vet Immunol Immunopathol.* Sep 10; 2002 87(3-4):147–60. [PubMed: 12072229]
- [33]. Yuan L, Josef C, Azevedo MS, Kim Y, Qian Y, Geyer A, et al. Protective immunity and antibody-secreting cell responses elicited by combined oral attenuated Wa human rotavirus and intranasal Wa 2/6-VLPs with mutant *Escherichia coli* heat-labile toxin in gnotobiotic pigs. *J Virol.* Oct; 2001 75(19):9229–38. [PubMed: 11533185]
- [34]. Yuan L, Azevedo MS, Gonzalez AM, Jeong KI, Van Nguyen T, Lewis P, et al. Mucosal and systemic antibody responses and protection induced by a prime/boost rotavirus-DNA vaccine in a gnotobiotic pig model. *Vaccine.* Jun 10; 2005 23(30):3925–36. [PubMed: 15917114]
- [35]. Ma Y, Qin M, Hu HQ, Ji G, Feng L, Gao N, et al. Immunogenicity of sabin inactivated poliovirus vaccine induced by diphtheria-tetanus-acellular pertussis and Sabin inactivated poliovirus combined vaccine. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi.* Jun; 2011 25(3):197–200. [PubMed: 21977591]
- [36]. Schutze MP, Leclerc C, Jolivet M, Audibert F, Chedid L. Carrier-induced epitopic suppression, a major issue for future synthetic vaccines. *J Immunol.* Oct; 1985 135(4):2319–22. [PubMed: 2411793]
- [37]. Adorini L, Harvey MA, Miller A, Sercarz EE. Fine specificity of regulatory T cells. II. Suppressor and helper T cells are induced by different regions of hen egg-white lysozyme in a genetically nonresponder mouse strain. *J Exp Med.* Aug 1; 1979 150(2):293–306. [PubMed: 88500]
- [38]. Schutze MP, Leclerc C, Vogel FR, Chedid L. Epitopic suppression in synthetic vaccine models: analysis of the effector mechanisms. *Cell Immunol.* Jan; 1987 104(1):79–90. [PubMed: 2433056]
- [39]. Zegers; Boersma. *Immunological Recognition of Peptides in Medicine and Biology.* Zegers, B.; Claassen, editors. CRC Press, Inc; Boca Raton, New York, London, Tokyo: 1995. p. 105-23.
- [40]. Santos N, Hoshino Y. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. *Rev Med Virol.* Jan-Feb; 2005 15(1):29–56. [PubMed: 15484186]
- [41]. Cunliffe NA, Gondwe JS, Broadhead RL, Molyneux ME, Woods PA, Bresee JS, et al. Rotavirus G and P types in children with acute diarrhea in Blantyre, Malawi, from 1997 to 1998: predominance of novel P[6]G8 strains. *J Med Virol.* Mar; 1999 57(3):308–12. [PubMed: 10022804]

Fig. 1. Analysis by SDS-PAGE and Western blot of purified recombinant proteins P2-P[8] VP8* (panels A and B) and P2-P[6] VP8* (panels C and D)

The proteins were expressed in *E. coli* at 18°C and purified as described in Materials and Methods. Lane M: molecular size markers. (panel A) SDS-PAGE analysis. Lane 1: purified P2-P[8] VP8*; and lane 2: purified P[8] VP8* as control. (panel B) Western blot analysis probed with guinea pig anti-Wa strain antiserum. Lane 1: purified P2-P[8] VP8*; and lane 2: purified P[8] VP8* as control. (panel C) SDS-PAGE analysis. Lane 1: purified P2-P[6] VP8*. (panel D) Western blot analysis probed with guinea pig anti-ST3 strain antiserum. Lane 1: purified P2-P[6] VP8*. Arrows indicate the recombinant proteins.

Fig. 2. Serum neutralizing antibody responses induced by P[8] VP8* and P[6] VP8* vaccines compared to P2-P[8] VP8* and P2-P[6] VP8* vaccines in guinea pigs

Guinea pigs were immunized IM with 10 μ g (n=4) or 20 μ g (n=4)/dose of the P[8] VP8*, P[6] VP8*, P2-P[8] VP8* or P2-P[6] VP8* vaccine with 100 μ g/dose of aluminum phosphate 3 times at 2-week intervals as described in Materials and methods. Because the antibody titers did not differ between 10 μ g and 20 μ g vaccine groups, the data were combined. Blood samples were collected immediately prior to each immunization and 7 days after the third immunization. The error bars represent standard errors of the means. Different capital letters on top of bars indicate significant differences between two vaccine groups at the same time (ANOVA-GLM, $P < 0.05$), while shared letters indicate no significant difference. Different numbers of * indicates significant difference after the first, second and third doses (ANOVA-GLM, $P < 0.05$), while same numbers of * indicate no significant difference.

Fig. 3. Addition of aluminum phosphate enhanced the immunogenicity of P2- VP8* vaccines (A, B) and kinetics of neutralizing antibodies induced by P2-P[8] VP8* vaccine in guinea pigs (C)

Guinea pigs were immunized IM with 10μg (n=4) or 20μg (n=4)/dose of P2-P[8] VP8* vaccine with or without 100μg/dose of aluminum phosphate; or 20μg (n=9)/dose of P2-P[6] VP8* vaccine with or without 100μg/dose of aluminum phosphate (panels A and B) as described in Materials and methods. Because the antibody titers did not differ between 10μg and 20μg vaccine groups, the data were combined. Blood samples were collected immediately prior to each immunization and 7 days after the third immunization. See Fig. 2 legend for statistical analysis and labeling.

The kinetics of neutralizing antibodies induced in guinea pigs by P2-P[8] VP8* vaccine (panel C). Guinea pigs were immunized IM with 10μg (n=4) or 20μg (n=4)/dose of the P2-P[8] VP8* vaccine with 100μg/dose of aluminum phosphate 3 times at 2-week intervals as described in Materials and methods. Blood samples were collected prior to each immunization as well as at 7 days or 2, 4 and 6 months after the third immunization.

Fig. 4. Serum antibodies and virus-specific T cell responses induced by the P2-P[8] VP8* vaccine in Gn pigs

Gn pigs were immunized IM with 50 μ g (n=5) of the purified P2-P[8] VP8* protein with 600 μ g/dose of aluminum phosphate or mock vaccinated (n=9) at PID 0, 10 and 21 and challenged with the virulent HRV Wa strain at PID 28. Serum samples were collected weekly from vaccinated and control pigs. Virus neutralizing antibody titers and Wa HRV-specific IgA antibody titers were compared between vaccinated and control pig groups on each time point (panels A and B); P[8] VP4-specific IgG antibody titers of the vaccinated pigs were compared among all the time points (panel C). Representative dot plots for detecting IFN- γ producing CD4+ and CD8+ T cells in the vaccinated and control pigs by flow cytometry are shown on the left of panel D. Frequencies of virus-specific IFN- γ producing T cells were compared between vaccinated and control pig groups at PCD 10 for the same cell type from the same tissue (right side of panel D). The error bars represent standard errors of the mean. Different letters on top of bars indicate significant difference between the pig groups (Kruskal-Wallis rank sum test; $P < 0.05$, $n = 3-5$); while shared letters or no letters indicate no significant difference.

Fig. 5. Clinical signs and virus shedding in P2-P[8] VP8* vaccinated Gn pigs postchallenge After challenge, the mean onset and duration of diarrhea and virus shedding (panel A), the mean accumulative fecal consistency scores (panel B) and the mean peak titers of virus shed (panel C) were calculated among all pigs in each group from PCD 0-7. The error bars represent standard errors of the mean. Different letters on top of bars indicate significant difference between the pig groups (ANOVA-GLM; $P < 0.05$), while shared letters indicate no significant difference

Table 1

Subunit P2-P[8] VP8* vaccine with aluminum phosphate adjuvant induced high levels of neutralizing antibodies to both P[8] and P[4] strains in guinea pigs

Rotavirus			Reciprocal of 60% PRN serum antibody titer ^a of 4 guinea pigs against indicated strains			
Strain	P type	G type	1	2	3	4
Wa	P[8]	1	5120	5120	10240	5120
P	P[8]	3	5120	1280	5120	5120
VA70	P[8]	4	5120	1280	5120	5120
WI61	P[8]	9	5120	1280	10240	1280
DS1	P[4]	2	1280	1280	5120	1280
1290	P[4]	8	640	1280	2560	1280
L26	P[4]	12	1280	1280	2560	1280
ST3	P[6]	4	160	160	640	160
1076	P[6]	2	160	160	320	160
69M	P[10]	8	80	40	80	80

^a 60% plaque reduction neutralizing antibody titer (reciprocal)