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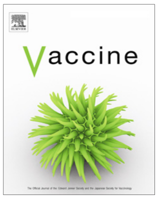

Protein deficiency reduces efficacy of oral attenuated human rotavirus vaccine in a human infant fecal microbiota transplanted gnotobiotic pig model

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ABSTRACT

Background: Low efficacy of rotavirus (RV) vaccines in developing African and Asian countries, where malnutrition is prevalent, remains a major concern and a challenge for global health.

Methods: To understand the effects of protein malnutrition on RV vaccine efficacy, we elucidated the innate, T cell and cytokine immune responses to attenuated human RV (AttHRV) vaccine and virulent human RV (VirHRV) challenge in germ-free (GF) pigs or human infant fecal microbiota (HIFM) transplanted gnotobiotic (Gn) pigs fed protein-deficient or -sufficient bovine milk diets. We also analyzed serum levels of tryptophan (TRP), a predictor of malnutrition, and kynurenine (KYN).

Results: Protein-deficient pigs vaccinated with oral AttHRV vaccine had lower protection rates against diarrhea post-VirHRV challenge and significantly increased fecal virus shedding titers (HIFM transplanted but not GF pigs) compared with their protein-sufficient counterparts. Reduced vaccine efficacy in protein-deficient pigs coincided with altered serum IFN-α, TNF-α, IL-12 and IFN-γ responses to oral AttHRV vaccine and the suppression of multiple innate immune parameters and HRV-specific IFN-γ producing T cells post-challenge. In protein-deficient HIFM transplanted pigs, decreased serum KYN, but not TRP levels were observed throughout the experiment, suggesting an association between the altered TRP metabolism and immune responses.

Conclusion: Collectively, our findings confirm the negative effects of protein deficiency, which were exacerbated in the HIFM transplanted pigs, on innate, T cell and cytokine immune responses to HRV and on vaccine efficacy, as well as on TRP-KYN metabolism.

1. Introduction

Rotavirus (RV) remains as a leading cause of childhood diarrhea worldwide. In 2000, RV caused an estimated 528,000 deaths in children under 5 years of age [1]. Due to global efforts since 2009, led by the World Health Organization (WHO) to introduce RV vaccines into routine childhood vaccination programs, estimated rotavirus deaths decreased to 146,000 in 2015 [2]. Vaccine efficacy against severe RV gastroenteritis was 85–98% in Latin American and European countries with mid to high socio-economic settings (SES). However, its efficacy was only 48–64% in African and Asian countries with low SES [3,4]. Although lower RV vaccine efficacy in low SES countries is consistently reported, little is understood about the biologic mechanisms underlying the vaccine underperformance.

Because malnutrition is prevalent in African and south Asian countries with low RV vaccine efficacy, we hypothesized that malnutrition will affect the immune responses to oral attenuated human RV (AttHRV) vaccination and subsequent virulent HRV (VirHRV) challenge [5]. Indeed, some clinical studies reported low protection rates of RV vaccine against RV diarrhea in malnourished children [6,7]. Malnutrition is a major contributor to the high

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mortality from viral gastroenteritis in low SES countries [4,5,8]. A number of field and animal studies have shown that malnutrition triggers immune dysfunction, including altered innate and adaptive immune responses, impairment of epithelial cell barriers and dysfunction of intestinal epithelial stem cells [9–14]. However, studies to elucidate the effect of malnutrition on an oral AttHRV vaccine are lacking.

There is an increasing interest in understanding the complex interrelationships among nutrition, gut microbiota, host immunity and enteric pathogens. The gut microbiota, through their metabolites and components such as lipopolysaccharide (LPS), polysaccharide A and formyl peptides, contributes to the host physiological and immunological functions such as nutrient absorption, development and maturation of the gut immune system, and protection against exogenous pathogens [15,16]. Nutrient availability impacts the composition and abundance of gut microbial taxa, and this in turn alters the nutritional metabolism of both the microbiota and the host, subsequently impacting the microbiota as well as host immunity and physiology [17–19]. Hence, physiologically relevant gnotobiotic (Gn) animal models that allow for nutritional and microbial manipulations are critical to assess and understand the interactions among nutrients, the microbiota and host immunity.

Tryptophan (TRP), an essential amino acid that cannot be synthesized de novo in humans, is reported to be a predictor of malnutrition [20,21]. TRP also plays an important role in immune regulation. After its absorption via the neutral amino acid transport B^0AT1 which is associated with angiotensin I converting enzyme 2 (ACE2), >90% of TRP is catabolized into kynurenine (KYN) via TRP 2,3-dioxygenase (TDO) in liver. However, upon infection and inflammation, extrahepatic TRP-KYN catabolism becomes dominant by inducing expression of indolamine 2,3-dioxygenase (IDO) on monocytes by stimulation with inflammatory cytokines such as IFN-α and IFN-γ [22,23]. Local TRP depletion and production of KYN via IDO suppress proliferation of effector T cells and NK cells, but enhance regulatory T cell activities [24–27]. IDO expression on dendritic cells (DCs) also induces further activation and differentiation of DCs [28]. Altered TRP homeostasis coinciding with decreased serum ACE2 levels has been observed previously in association with VirHRV challenge of Gn pigs fed a protein deficient diet [10]. However, the interrelationships between TRP-KYN metabolism and T cell and cytokine responses to RV vaccination and challenge are unclear.

We previously established a protein-deficient human infant fecal microbiota (HIFM)-transplanted neonatal Gn pig model that recapitulates major aspects of protein malnutrition in children [9,10]. The aim of this study was to understand the immunologic and biologic mechanisms underlying the reduced vaccine efficacy in developing countries in the context of childhood protein deficiency. Here, using this HIFM-transplanted Gn pig model on protein sufficient or deficient bovine milk diet, we assessed the efficacy of the AttHRV oral vaccine against VirHRV challenge and compared innate, T cell and cytokine immune responses as well as TRP-KYN metabolism. We also included non-HIFM transplanted germ-free (GF) counterpart groups to elucidate the immunomodulating effects of the transplanted HIFM on protein deficiency and on the other study parameters.

2. Materials and Methods

2.1. Human infant fecal microbiota (HIFM)

The collection and use of HIFM were approved by The Ohio State University Institutional Review Board. With parental consent, sequential fecal samples were collected from a healthy, two-month-old, exclusively breastfed, vaginally delivered infant. Samples were pooled and diluted to 1:20 (wt/vol) in phosphate buffer solution containing 0.05% (vol/vol) cysteine and 30% glycerol and stored at −80 °C as described previously [9,10].

2.2. Virus

The cell-culture adapted attenuated HRV (AttHRV) Wa G1P [8] strain passaged in African green monkey kidney cells (MA104) was used as a vaccine at a dose of 1×10^7 fluorescent foci-forming units (FFU) [29]. The Gn pig passaged virulent HRV (VirHRV) Wa strain at pig passages 25–26 was used as challenge virus at a dose of 1×10^6 FFU as described previously [9,10].

2.3. Animal experiments

The animal experiments were approved by the Institutional Animal Care and Use Committee at The Ohio State University. Piglets were derived from near-term sows (purchased from OSU specific pathogen-free swine herd) by hysterectomy and maintained in sterile isolators [30]. Neonatal pigs obtained from five litters (5–15 pigs/litter) were randomly assigned to one of four groups: (1) protein deficient diet, GF (no HIFM) (Deficient group, n = 12); (2) protein sufficient diet, GF (no HIFM) (Sufficient group, n = 11); (3) protein deficient diet, HIFM transplanted (Deficient HIFM group, n = 12); and (4) protein sufficient diet, HIFM transplanted (Sufficient HIFM group, n = 11). Pigs in Sufficient GF and Sufficient HIFM groups were fed 100% ultra-high temperature pasteurized bovine milk (Parmalat) that met or exceeded the National Research Council Animal Care Committee's guidelines for calories, fat, protein and carbohydrates in suckling pigs. Pigs in Deficient GF and Deficient HIFM groups were fed 50% Parmalat and 50% sterile water, which contained half of the recommended protein levels (7.5% vs 15% of diet). All pigs were confirmed as free from bacterial and fungal contamination prior to HIFM transplantation by aerobic and anaerobic cultures of rectal swabs. Pigs in Deficient HIFM and Sufficient HIFM groups were orally inoculated with 2 ml of diluted HIFM stock at 4 days of age (post-HIFM transplantation day, PTD 0). Rectal swabs were collected once or twice a week to analyze the microbial compositions by 16S metagenomic analysis as described previously [9,31]. All pigs were orally vaccinated twice at a 10-days interval with AttHRV at PTD 7/post-1st vaccination day, PVD 0 and PTD 17/PVD 10 [post-2nd vaccination day 0, thereafter referred as PVD10 (0)]. At PTD 24/PVD 17 (7)/post-challenge day (PCD) 0, a subset of pigs from each of the four group was euthanized to assess vaccine responses pre-challenge. The remaining pigs were challenged with VirHRV and euthanized at PTD 31/PVD 24 (14) /PCD 7.

2.4. Assessment of clinical signs and detection of HRV shedding

Rectal swabs were collected daily post-challenge. Fecal consistency was scored as follows; 0, normal; 1, pasty/semi-liquid; and 2, liquid, and pigs with fecal score more than 1 were considered as diarrheic. Rectal swabs were suspended in 2 ml of minimum essential medium (MEM) (Life technologies, Waltham, MA, USA), clarified by centrifugation for 800*g* for 10 min at 4 °C, and stored at −20 °C until quantitation of infectious HRV by cell culture immunofluorescence assay as previously described [32].

2.5. Isolation of MNCs and flow cytometry

Blood, spleen, ileum and duodenum were collected to isolate mononuclear cells (MNCs) as described previously. Freshly isolated MNCs were stained for determining T cell subsets: T helper ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$), natural and inducible regulatory T cells ($CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^+$, respectively) and activated T cells ($CD4^+CD25^+Foxp3^-$ and $CD8^+CD25^+Foxp3^-$) [33]. To determine the frequencies of HRV-specific

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