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Oral retinyl palmitate or retinoic acid corrects mucosal IgA responses toward an intranasal influenza virus vaccine in vitamin A deficient mice

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Abstract

Vitamin A deficiency (VAD) is a leading cause of pediatric morbidity and mortality due to infectious diseases. Recent pre-clinical studies have revealed that VAD impairs mucosal IgA-producing antibody forming cell (AFC) responses toward a paramyxovirus vaccine in the upper respiratory tract (URT), thus impeding a first line of defense at the pathogen's point-of-entry. The studies described here tested the hypothesis that VAD may also impair immune responses after FluMist vaccinations. Results show that (i) IgA-producing antibody forming cells (AFCs) are significantly reduced following FluMist vaccination in VAD mice, and (ii) oral doses of either retinyl palmitate or retinoic acid administered on days 0, 3, and 7 relative to vaccination rescue the response. Data encourage the conduct of clinical studies to identify FluMist vaccine weaknesses in human VAD populations and to test corrective supplementation strategies. Improvements in vaccine efficacy may ultimately reduce the morbidity and mortality caused by influenza virus worldwide.

Keywords

FluMist; respiratory tract; mucosal IgA; oral; retinyl palmitate; retinoic acid; vitamin A; antibody forming cells

Introduction

Vitamin A deficiencies (VAD) and insufficiencies have been long-standing problems in both developed and developing countries, particularly in conditions of low socioeconomic status,

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prematurity and disease. VAD is best known for its contribution to xerophthalmia, while its contribution to infectious diseases is less well appreciated. Individuals with low serum levels of retinol or retinol binding protein are especially vulnerable to morbidity and mortality caused by gastrointestinal and respiratory viral infections [1-3].

To prevent infectious diseases of the respiratory tract, we are developing Sendai virus (SeV) as a candidate vaccine for human parainfluenza virus type 1 and as a recombinant vaccine for respiratory syncytial virus (RSV [4]). Each of these vaccines is administered intranasally (i.n.), as is FluMist, a vaccine licensed by MedImmune (Gaithersburg, MD) and produced each year in response to drifting and shifting influenza virus variants. FluMist comprises a cocktail of reassortant, replication-competent, cold-adapted influenza viruses that express internal proteins from a master donor virus, and hemagglutinin and neuraminidase membrane proteins from circulating wildtype influenza viruses. FluMist is recommended for vaccination of individuals between the ages of 2 and 49 years of age by i.n. spray. The vaccine is not recommended for use in very young children, the elderly, and individuals with asthma, due in part to potential adverse events including hospitalization and wheezing. The i.n. administration of vaccines ensures the induction of a robust IgA antibody forming cell (AFC) response in the upper respiratory tract (URT)[5], a first line of defense against virus at its point-of-entry, and a correlate of protection against viral disease [6-9].

Using a mouse model for VAD, we previously demonstrated that SeV-specific mucosal IgA responses were impaired in the URT of VAD animals [10]. We therefore asked if VAD would also affect responses toward FluMist. Results in this report show that the IgA-AFC response in the URT was indeed impaired toward FluMist in VAD animals. However, the administration of vitamin A supplements at the time of vaccination was sufficient to reverse the defect and induce healthy levels of FluMist-specific IgA AFCs. Results encourage clinical evaluation of FluMist responses in children in the context of VAD, and the testing of vitamin A supplements to improve FluMist vaccine efficacy in VAD populations.

Materials and Methods

VAD mice and vaccinations

Pregnant female C57BL/6 mice were from Jackson Laboratories (Bar Harbor, Maine). Animals were housed in a Biosafety Level 2⁺ area as specified by AAALAC and approved by the IACUC. To establish VAD mice, day 4-5 estrus C57BL/6 females were placed on VAD or control diets (the latter diet was supplemented with 15 IU Vitamin A palmitate/gram; Harlan Laboratories, Madison, WI) upon arrival at St. Jude Children's Research Hospital, as described previously [10;11]. The standard rodent breeder diet at St. Jude contains 30 IU/gram vitamin A (Autoclavable Rodent Breeder Diet #5013; LabDiet, Brentwood, MO). Sera were spot-checked for retinol by submission to the Colorado State Veterinary Laboratory for retinol testing at the Texas Veterinary Diagnostic Laboratory (Fort Collins).

Viral infections and vitamin supplementation

Infections of adult mice involved anesthesia followed by i.n. inoculations with FluMist (30 µl undiluted FluMist; seasons 2011-12 or 2012-13). This high dose was used, because the licensed, human vaccine had not been mouse-adapted. VAD animals were grouped to receive oral supplements on days 0, 3 and 7 (relative to vaccination) by gavage with either retinoic acid (300 µg/mouse; Sigma Cat#R2625) in 100 µl canola oil or retinyl palmitate (600 IU/mouse; Nutrisorb A, Interplexus Inc., Kent, WA) in 100 µl PBS. The numbers of mice per group are stated in figure legends. All immunizations and downstream analyses were repeated to ensure reproducibility.

Preparation of samples

Animals were sacrificed one month after vaccinations with avertin and exsanguination. Nasal wash samples were collected by exposing the trachea and washing the upper trachea and nasal cavity with 200 µl of PBS. Mice were perfused with PBS injected through the retro-orbital sinus. Diffuse nasal-associated lymphoid tissues(d-NALT) were harvested as described previously [10;11].

ELISA

The influenza virus-specific ELISA was conducted by plating Fluzone (Sanofi Pasteur) matched by season with vaccine on 96-well plates. Prior to plating, Fluzone was mixed with disruption buffer (500 µl Fluzone + 40 µl PBS + 60 µl disruption buffer (0.05% TritonX-100, 60 mM KCl, 10 mM Tris pH7.8)) and brought to a final concentration of 1 µg/ml in PBS. After blocking plates, serially diluted test samples were applied for 1h incubation at 37°C. Plates were washed and developed with alkaline phosphatase-conjugated goat anti-mouse IgA (Cat #1040-04, Southern Biotechnology, Birmingham, AL) followed by p-nitrophenyl phosphate (Sigma Aldrich Cat#N2640) for reading at OD 405 nm.

AFC ELISPOT

ELISPOT plates (multiscreen™-IP, Millipore, Cat#MAIPS4510, Billerica, MA) were coated with Fluzone (Sanofi Pasteur) as described previously [5] and incubated overnight at 4°C. Wells were washed with PBS and blocked with medium containing 10% FCS. Cells were then applied to wells (1×10⁵ cells/well) usually in triplicate per sample and incubated for 3 hours at 37°C. Plates were washed and incubated with 100 µl of alkaline phosphatase-conjugated goat anti-mouse IgA (Cat #1040-04) in PBS-Tween 20 with 1% BSA. After overnight incubation at 4°C, antibodies were removed and plates were developed with BCIP/NBT substrate (Sigma Aldrich, Cat#B5655) and rinsed with water prior to spot counting.

Statistics

Unpaired T and Fisher's exact tests were used to compare groups (GraphPad Prism, San Diego, CA).

Results and Discussion

VAD impairs URT mucosal IgA responses toward FluMist vaccinations

Based on our previous results showing that mucosal IgA responses toward a candidate parainfluenza virus vaccine (SeV) were impaired in a VAD mouse model, we questioned whether IgA responses toward FluMist might also be poor. To initiate the study, we placed pregnant females on VAD or control diets. The control diet matched the VAD diet precisely except for the addition of 15 IU/gram vitamin A palmitate. Progeny were also maintained on VAD or control diets until adulthood when experiments were conducted. As shown in Figure 1A, animals on a standard rodent breeder diet (LabDiet) or the control diet (Harlan) exhibited normal levels of serum retinol ($20 \mu\text{g/dL}$ [12;13]), whereas animals on the VAD diet (Harlan) had undetectable serum retinol levels, confirming their VAD status.

Adult VAD and control animals were administered FluMist vaccine and sacrificed one month later for the analysis of virus-specific IgA-producing AFCs in the URT. As shown by a representative experiment in Figure 1B, virus-specific IgA-producing AFC were significantly reduced in the d-NALT of VAD animals, precisely as had been observed in SeV experiments. Also similar to previous experiments, serum virus-specific IgG levels were not significantly impacted by VAD (data not shown)[10;14].

We next tested the hypothesis that an oral dosing of vitamin A at the time of vaccination might reverse the loss of vaccine-induced IgA-producing AFC in the URT mucosa. To this end, we first administered vitamin A in the form of retinoic acid (RA) by gavage on days 0,3, and 7 relative to vaccination with FluMist. As demonstrated in Figure 2A, the IgA responses in the VAD nasal washes were low, but IgA in the nasal wash was higher in VAD mice treated with RA (Figure 2A). Furthermore, there was a statistically significant increase in the number of IgA-producing AFC in the d-NALT of vitamin-supplemented VAD animals (Figure 2B).

Given that RA is not a preferred form of oral supplement in the clinical arena, we conducted additional experiments in which RA was replaced by retinyl palmitate. As shown in Figure 2C, IgA in the nasal wash was again improved in treated VAD mice. Most importantly, the URT d-NALT IgA-producing AFCs, responsible for IgA production at the mucosal surface, were significantly and repeatedly improved, exhibiting levels typical of control, healthy animals (Figure 2D).

A final question was whether the FluMist vaccine could prevent an influenza virus challenge in mice and whether VAD mice exhibited impaired protection compared to control animals. An initial test demonstrated that FluMist was not protective against a mouse adapted PR8 virus challenge in either VAD or control animals. This was not surprising as FluMist is a human vaccine that has not been adapted for amplification in rodents. As an alternative strategy for comparing vaccine efficacy in VAD and control animals, we immunized mice with a mouse-adapted, cold-adapted derivative of A/Puerto Rico/8/34 (PR8) virus, which was kindly provided to us by Drs. Huber and McCullers[15;16]. In a preliminary experiment, we demonstrated that IgA AFC responses were significantly impaired in VAD mice compared to control mice that received the cold-adapted vaccine. Control and VAD

mice were also immunized with a dose of approximately 2.5×10^7 EID₅₀ cold-adapted vaccine and challenged one month later with wildtype PR8 (approximately 1×10^4 EID₅₀ per mouse). In one of two experiments, there was a significant difference between challenge virus amplification in VAD mice compared to controls ($p < .05$, Fisher's exact test). All control mice (10/10) were fully protected from infection with the challenge virus whereas only a fraction of VAD mice (3/6) were fully protected. Experiments are in progress to study the effects of VAD and vitamin supplementation on the protection conferred by additional respiratory virus vaccines in mice.

IgA and protective immunity

Results from this study demonstrated the effects of VAD on the induction of mucosal IgA responses following i.n. influenza virus vaccination. Previous studies have revealed the importance of IgA as a correlate of protection against respiratory virus infections [6-9], although a comprehensive study of IgA functions has not been conducted. While IgA is best known for its binding and neutralizing capacities, this antibody isotype is responsible for numerous additional functions that include modulation of cellular and cytokine immune effectors [17-21]. Future experiments are warranted to analyze the full impact of IgA and other isotypes on respiratory virus control, before and after [22] the infection of mammalian cells.

Vitamin A supplementation; efficacy and toxicity considerations

Decades ago, it was established that vitamin A supplements could protect VAD children from the morbidity and mortality caused by measles virus [23], and the World Health Organization established recommendations that supplements be administered orally to children at the time of routine measles vaccinations. The recommended dose of vitamin A for this purpose is 100,000 IU in infants and 200,000 IU in children 12 months or older [24]. Vitamin A palmitate is used as a preferred metabolite based on its safety and stability profile compared to retinoic acid. However, some toxicities are associated with high doses of vitamin A even when vitamin is administered in palmitate form. The precise human dose required to ensure efficacy without toxicity remains a point of debate. Studies have shown that doses between 100,000- 400,000 IU vitamin A associate with significant protection from morbidity and mortality in children with measles [23;25]. Lower doses (<50,000 IU) have been recommended to reduce the risks of acute side effects, but the benefit of lower doses is not always evident [26-29]. Clinical results differ depending on the regimen of supplementation and the degree of VAD in the target population. In our study, we tested 600 IU/~20g VAD mouse, a relatively high dose (comparable to 300,000 IU in a 12 month old child of 10 kg). We are currently conducting additional murine experiments to define the lowest retinyl palmitate dose, preferably delivered only once during vaccination, sufficient to correct the d-NALT IgA-producing AFC response.

While mouse models provide proof-of-concept information, clinical studies of FluMist vaccine efficacy in individuals of known retinol status would be most informative to determine if VAD associates with impaired responses in humans. If an association is realized, vitamin A supplementation studies might then determine if weak immune responses in VAD hosts can be corrected. Programs directed toward a logistically-feasible

improvement in URT responsiveness toward respiratory virus vaccines may enhance an important correlate of protection and thereby reduce the morbidity and mortality caused by respiratory virus infections worldwide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The VAD mouse model exhibits an impaired mucosal IgA AFC response toward FluMist

Panel A: Prior to FluMist vaccinations, mice on a control or VAD diet (Harlan Laboratories; see Materials and Methods) were compared to mice on a standard diet (Rodent Breeder Diet #5013 produced by LabDiet) for serum retinol levels. Each bar represents results from an individual mouse. Panel B: One month following FluMist vaccinations, mice on control (n=3) and VAD (n=3) diets were tested for virus-specific IgA-producing AFCs in the d-NALT with an ELISPOT assay.

Figure 2. Retinoic acid and retinyl palmitate rescues impaired mucosal IgA AFC responses toward FluMist in the VAD mouse

Panel A: Control mice (n=3), VAD mice (n=2), and RA-supplemented VAD mice (n=3) were tested for mucosal IgA by ELISA. Panel B: Control mice (n=5), VAD mice (n=3), and RA-supplemented VAD mice (n=5) were tested for d-NALT IgA-producing AFCs with an ELISPOT assay. Panel C: Control mice (n=5), VAD mice (n=2), and retinyl palmitate -supplemented VAD mice (n=5) were tested for mucosal IgA. Panel D: Control mice (n=5), VAD mice (n=5), and retinyl palmitate -supplemented VAD mice (n=5) were tested for d-

NALT IgA-producing AFCs. Some mortality of VAD mice during experiments yielded the variable group sizes described above.