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Research paper

Protein energy malnutrition alters mucosal IgA responses and reduces mucosal vaccine efficacy in mice

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ABSTRACT

Oral vaccine responsiveness is often lower in children from less developed countries. Childhood malnutrition may be associated with poor immune response to oral vaccines. The present study was designed to investigate whether protein energy malnutrition (PEM) impairs B cell immunity and ultimately reduces oral vaccine efficacy in a mouse model. Purified isocaloric diets containing low protein (1/10 the protein of the control diet) were used to determine the effect of PEM. PEM increased both nonspecific total IgA and oral antigen-specific IgA in serum without alteration of gut permeability. However, PEM decreased oral antigen-specific IgA in feces, which is consistent with decreased expression of polymeric Immunoglobulin receptor (pIgR) in the small intestine. Of note, polymeric IgA was predominant in serum under PEM. In addition, PEM altered B cell development status in the bone marrow and increased the frequency of IgA-secreting B cells, as well as IgA secretion by long-lived plasma cells in the small intestinal lamina propria. Moreover, PEM reduced the protective efficacy of the mucosally administered cholera vaccine and recombinant attenuated *Salmonella enterica* serovar Typhimurium vaccine in a mouse model. Our results suggest that PEM can impair mucosal immunity where IgA plays an important role in host protection and may partly explain the reduced efficacy of oral vaccines in malnourished subjects.

1. Introduction

Protein energy malnutrition (PEM) continues to be a major health concern in developing countries, particularly in southern Asia and Africa [1–3]. Children with insufficient and inadequate protein consumption are more prone to infections and endemic diseases. In order to reduce the prevalence of diseases related to secondary immune deficiency in children with PEM, various strategies have been introduced including appropriate nutrition and vaccination policies and improved sanitation and hygiene [4].

Several studies have reported that the efficacy of most licensed oral vaccines (*e.g.*, rotavirus, poliovirus, and cholera) is relatively low in children of developing countries compared to those of developed countries [5,6]. Oral rotavirus vaccine efficacy in infants from industrialized countries is more than 90% but in developing countries it is just 30%–40% [7]. Seroconversion rates following oral vaccine administration affects the vaccine's efficacy. Children given oral poliovirus vaccine in developed countries have seroconversion rates of nearly 100% compared with 70%–73% in developing countries [8,9]. Two different oral cholera vaccines have been approved by the World Health Organization, Dukoral® (Crucell, Netherlands) and Shanchol™ (Shantha Biotechnics, Hyderabad, India). Dukoral® provides 85% protection against *Vibrio cholerae* O1 in children 2–15 years old, based on the previous study which was done in Bangladesh before it was licensed [10]. The cumulative protective efficacy of Shanchol™ at 5 years after vaccination in Kolkata, India was 65% with a lower efficacy in younger children [11]. A different study of oral cholera vaccination (OCV-023, 024 and 028) of children found antibody increases in 86% of Swedish children but in only 36%–45% of Nicaraguan children [12].

Decreased oral vaccine efficacy is associated with poor mucosal

Abbreviations: CT, cholera toxin; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; HPLC, high performance liquid chromatography; LPD, low protein diet; LPL, lamina propria lymphocytes; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cell; PEM, protein energy malnutrition; pIgR, polymeric immunoglobulin receptor; RASV, recombinant attenuated salmonella vaccine strain *Salmonella enterica* serovar Typhimurium χ9241; SFC, spot forming cells

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immunogenicity of the vaccine in the gut, which may induce increased susceptibility to bowel infections or even death. For children who live in areas in which cholera is endemic, PEM is one of the factors that could contribute to poor vaccine responsiveness [12,13].

PEM increases IgA in serum but decreases intestinal polymeric immunoglobulin receptor (pIgR) [14,15]. Because pIgR mediates transport of polymeric IgA through the mucosal epithelia, decreased pIgR may imply low concentration of IgA in intestinal mucous secretions.

The present study was designed to investigate whether PEM affects impaired mucosal B cell immunity and oral vaccine efficacy in a mouse model. Purified isocaloric diets containing 2% protein were used to determine the effect of a low protein diet on the secretion and the molecular conformation of IgA in mucosal tissues as well as on pIgR expression. The protective efficacy of a killed oral cholera vaccine and of a recombinant attenuated salmonella oral vaccine, *Salmonella enterica* serovar Typhimurium χ9241 (RASV), was also evaluated [16].

2. Materials and methods

2.1. Mice and experimental diets

Female C57BL/6 mice were purchased from Charles River Laboratories (Orient Bio, Seoul, Korea). Purified isocaloric diets containing 2% (low protein diet; LPD) or 20% protein (control diet) were manufactured and purchased from Dae han BioLink CO. (Chungbuk, Korea). The composition and caloric content of the diets are shown in Tables 1 and 2 in Supplementary material. Eight-week-old mice were fed the diets for 6 weeks *ad libitum*. Individual mouse weights were measured every week. All animal experiments were approved by the Institutional Animal Care and Use Committee at the International Vaccine Institute and performed according to legal requirements.

2.2. Blood analysis for protein and cholesterol levels

Blood from each mouse was obtained after 6 weeks of diet. Serum was collected following centrifuging at 2500 rpm for 20 min. Samples were analyzed for total protein (g/dl), albumin (g/dl), and total cholesterol (mg/dl) by the Gyeonggi Institute of Science & Technology Promotion (GSTEP; Gyeonggido, Korea).

2.3. Analysis of serum, feces, and intestinal wash samples

Blood, feces and small intestines from each mouse were collected after 6 weeks of diet, and IgA, IgG, pIgR and antigenic specificity were determined by ELISA. Serum was prepared from blood obtained *via* the retro-orbital plexus. Two or three fecal pellets were collected from each mouse and PBS (0.1 g/1 ml) was added with protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA). Fecal samples were vortexed at 4 °C overnight and then centrifuged at 13,000 rpm for 5 min. The supernatants were used for ELISA. Intestinal fluid was obtained by passing 3 ml of PBS containing protease inhibitor cocktail (Cell Signaling Technology) through the small intestine of each mouse.

Each 96-well immunoplate (Maxisorp; Nunc, Roskilde, Denmark) was coated with purified goat $F(ab')_2$ anti-mouse IgM + IgG + IgA antibody (200 ng/well in 100 µl of PBS; SouthernBiotech, Birmingham, AL), attenuated cholera toxin (CT) (200 ng/well in 100 µl of PBS; List Biological Laboratories, Campbell, CA), *S.* Typhimurium derived LPS (500 ng/well in 100 µl of PBS; Sigma Aldrich, St. Louis, MO), or mouse pIgR antibody (100 ng/well in 100 µl of PBS;) and incubated all at 4 °C overnight. Each well was washed four times with PBS containing 0.05% Tween®20 (Sigma Aldrich) and blocked with 100 µl of 1% Bovine serum albumin (InvivoGen, San Diego, CA) dissolved in PBS for 1 h at room temperate (RT). After being washed, 100 µl of serially diluted sera and fecal extract or 50 µl of serially diluted intestinal wash in blocking buffer was added and the plates were incubated for 2 h at RT. Subsequent washing, 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA or IgG (SouthernBiotech) was diluted to 1/6000 in blocking buffer or 50 µl of goat anti-mouse pIgR (R & D Systems, Minneapolis, MN) was diluted to 1/400 in blocking buffer and added to each well and incubated for 1 h at RT. As detection antibody for pIgR, we used 50 µl of HRP-conjugated rabbit anti-goat IgG (1/5000; R & D Systems). After being washed, 50 or 100 µl of peroxidase substrate solution (TMB; Moss, Pasadena, MD) was added and the plates were allowed to stand for 20 min in the dark. After 50 µl of 0.5N HCL was added to each well to stop the reaction, the absorbance at 450 nm was measured by microplate reader (SpectraMax 340PC 384; Molecular Devices, Sunnyvale, CA) and the antibody titer was expressed as reciprocal $\log_2$ of dilutions showing 0.2 values.

2.4. ELISPOT assay

Single cells of spleen, mesenteric lymph nodes, and Peyer's patches (PP) were isolated from the mice by passing tissues through a 70-µm nylon cell strainer (BD, Franklin Lakes, NJ). Bone marrow cells were prepared from leg bones of each mouse by flushing the femurs and tibias by inserting a needle-attached syringe filled with Roswell Park Memorial Institute (RPMI) 1640 medium (PAA Laboratories, Pasching, Austria) at the end of the bone until the bone became white. Peripheral blood mononuclear cells (PBMC) were collected using cell separation media (Lympholyte; Cedarlane, Burlington, Canada). Lamina propria lymphocytes (LPL) were prepared from small intestines by treatment with EDTA (10 or 2.5 mM for mice given 20% and 2% diets, respectively; Gibco, Grand Island, NY) on a magnetic stirrer for 20 min at 37 °C. After extensive washing with PBS, the tissues were incubated with RPMI 1640 medium (PAA) containing collagenease D (0.5 mg/ml) and DNase I (0.1 mg/ml; both Roche, Mannheim, Germany) for 45 min at 37 °C with stirring. Single-cell suspensions were obtained by passing through a 70-µm nylon cell strainer (BD). Following centrifugation, the pellets were resuspended with RPMI 1640 containing 10% FBS.

Each 96-well nitrocellulose microplate (MultiScreen HTS™-HA; Millipore, Molsheim, France) was coated with purified goat $F(ab')_2$ anti-mouse IgM + IgG + IgA antibody (200 ng/well in 100 µl of PBS; SouthernBiotech) and incubated these at 4 °C overnight. After wells were washed twice with PBS, each well was blocked with 150 µl of RPMI 1640 containing 10% FBS for 1 h at 37 °C. The plates were washed with PBS and 50 µl of alkaline phosphatase (AP)-conjugated goat anti-mouse IgA or IgG (1:1000) (SouthernBiotech) was added to each well, followed by the addition of serially diluted single cell suspensions (5×10^5 cells, 2-fold dilution, 50 µl/well). After another incubation (4 h at 37 °C in 5% CO2), the plates were washed extensively seven times with PBS and three times with PBS containing 0.05% Tween®20, soaked for 5 min, and washed with deionized water. Spots were developed by adding 80 µl of BCIP®/NBT liquid substrate (Sigma Aldrich) and counted by ImmunoSpot analyzer (Cellular Technology, Cleveland, OH).

2.5. Flow cytometric analysis

Single cells from splenocytes, mesenteric lymph nodes, LPL and bone marrow were isolated as described above. The following conjugated antibodies (all from BD) were used: anti-CD45R/B220, anti-CD3e, anti-CD8b, and anti-CD138. Data were acquired on an LSR II (BD) flow cytometer and analyzed using FlowJo (Ashland, OR) software.

2.6. High performance liquid chromatography (HPLC)

Mouse blood was collected after 6 weeks on diet and pooled serum samples from each group were prepared. In order to separate different molecular forms of IgA, size fractionation was performed by HPLC on Superdex 200 10/300 GL columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; sample volume: 500 µl; eluent: 0.05 M phosphate