Retinol and Retinol-Binding Protein: Gut Integrity and Circulating Immunoglobulins

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Vitamin A (retinol) is required to maintain immunity and epithelial turnover and is a key micronutrient needed for combating infection. Vitamin A actions on the immune system are diverse and cannot be accounted for by a single effect or mechanism. The actions of retinol in maintaining gut integrity in humans and immunoglobulin levels in mice was investigated. For 30 children, performance on the lactulose/mannitol test, a test commonly used to assess intestinal barrier function, was inversely correlated (P = .012) with serum retinol concentrations. Thus, children with lower serum retinol, and presumably poorer vitamin A nutritional status, are more likely to have impaired intestinal integrity. Knockout mice that have impairments in plasma retinol transport have circulating immunoglobulin levels that are half those observed in matched wild type mice. No differences were observed in B and T cell populations present in spleen, thymus, and bone marrow.

Vitamin A (retinol) is acquired from the diet and is needed for maintaining the general health of higher animals and humans [1]. It is required for normal vision, for maintaining normal growth and differentiation, for an uncompromised immune response, and for numerous other essential biologic processes [1–5]. Following its uptake in the small intestine from the diet, vitamin A is packaged as retinyl ester along with other dietary lipids in chylomicrons and secreted into the lymphatic system [6, 7]. Postprandial vitamin A is taken up by the liver and is either stored in hepatic reserves or secreted boud to retinol-binding protein (RBP) to meet tissue needs for vitamin A. The only known physiologic function of RBP is to deliver retinol to tissues [1, 7]. RBP circulates in the blood as a 1:1 molar complex with another serum protein, transthyretin (TTR) (formerly called prealbumin) [1, 7]. Normal levels of RBP in human serum are ~40–60 μg/mL (~2–3 μM). In rodents, RBP levels in serum are 20–30 μg/mL (~1–1.5 μM). Within individual humans or rodents, RBP levels are highly regulated and remain constant, except in extremes of vitamin A nutriture or in disease.

It is generally believed that the predominant route through which most tissues acquire the vitamin A they need for regulating gene expression is via the intracellular oxidation of retinol to retinoic acid [6]. Since RBP is the sole plasma transporter of retinol, RBP has been assumed to play an essential role in providing tissues with the vitamin A needed for regulating retinoic acid–responsive gene expression. The all-trans and 9-cis isomers of retinoic acid, the major active forms of vitamin A, act as ligands for transcription factors that modulate expression of a large number of genes, including those encoding hormones, growth factors, transcription factors, membrane receptors, extracellular matrix proteins, structural proteins, and enzymes involved in diverse metabolic processes [4, 5]. The transcriptional regulatory actions of all-trans- and 9-cis retinoic acid are mediated by members of the retinoic acid receptor and retinoid X receptor (RXR) families of ligand-dependent transcription factors [4]. Since members of the RXR receptor family can serve as partners in forming heterodimers with the vitamin D receptor, the thyroid hormone receptors, the peroxisomal proliferator activator receptors, and several other ligand-dependent transcription factors, 9-cis retinoic acid likely plays an important role in regulating a broad-spectrum of hormonally responsive genes [4].

Vitamin A deficiency (VAD) persists as a problem of significant public health concern in many regions of the world [8] and is associated with serious increases in morbidity and mortality [8–15]. The World Health Organization estimates that
about 250 million preschool-aged children suffer from moderate-to-severe VAD [8, 9]. Children experiencing VAD are especially prone to infection, particularly infections of the lung and gastrointestinal tract [8, 9, 12]. Ultimately, the increased morbidity and mortality associated with VAD in children arise from infection. Our understanding of the actions of vitamin A in maintaining a healthy immune system is still relatively limited [16, 17], especially of the effect of vitamin A on the circulatory and immune functions in humans and animals [16, 17].

Because we wish to know how vitamin A nutritional status influences the gut integrity and immune response in humans and animals, we explored the actions of vitamin A in maintaining gut integrity in infants and young children and investigated linkages between vitamin A and the immune response in induced mutant mice. Results from our pilot studies are described below.

Methods

Subjects. Subjects in this study are part of a cohort of children from an urban slum community (Gonçalves Dias) of ~2000 inhabitants near the medical center of the Federal University of Ceará in Fortaleza, Brazil. These children have been followed since birth with 2 visits per week from a study nurse and 2 health care workers [18]. Full descriptions of this cohort of children have been published previously as the cohort has been the focus of studies of diarrheal and other infectious diseases [18]. From this cohort, 30 mildly malnourished children, ranging in age from 1 to 9 years, participated in the current study. At the time of study, children showed no signs of infection or other obvious illness as assessed by a physical examination performed by a physician or physician’s assistant.

Lactulose/mannitol test. The lactulose/mannitol test was done by procedures that are well established and reported in the literature [19, 20]. In brief, after an overnight fast, each subject drank a solution of 5 g of lactulose (4-O-D-galactopyranosyl-D-fructofuranose; obtained from Duphar, Worden, The Netherlands) and 1 g of mannitol (6-O-D-galactopyranosyl-D-glucopyranose; obtained from Quimiobras, Rio de Janeiro) dissolved in 20 mL of water. After 1 h, subjects were encouraged to drink to maintain good urine output. They ate ad libitum. Urine was collected over a 5-h period in a container with 1 mL 20% (wt/vol) chlorohexidine as a preservative. Total urine volume was recorded upon completion of the test, and a 20 mL aliquot was stored at −20°C until analysis.

We used a high-performance liquid chromatography (HPLC) procedure employing pulsed amperometric detection to determine lactulose and mannitol levels in the urine [19]. This procedure is simple and sensitive and enables simultaneous determination of lactulose and mannitol from the same urine sample. In brief, 50 μL of urine was diluted in 3 mL of water, filtered by centrifugation through a 0.22-μm cellulose acetate membrane (Spin-X centrifuge filter unit; Costar, Cambridge, MA), and 50 μL of the filtrate was injected onto the HPLC column. Chromatography was done on a 250- × 4.0-mm CarboPac MA-1 anion exchange column (Dionex, Sunnyvale, CA) connected to a BioLC carbohydrate analyzer HPLC system (Dionex). The column was eluted isocratically by using 480 mM NaOH in water as solvent flowing at 0.4 mL/min at ambient temperature. For detection, a pulsed amperometric detector was used. The sensitivity, linearity, and resolution of this procedure for mannitol and lactulose have been documented [19]. The lactulose/mannitol permeability test was considered abnormal or positive for comparison purposes if the lactulose/mannitol ratio was 8.030 [20–24].

Serum retinol determinations. Serum retinol levels were measured by reverse-phase HPLC as previously described [25]. This method employs an internal standard consisting of a known mass of retinyl acetate. The amount of retinol present in each extracted serum sample was quantitated by comparisons of the integrated areas under the HPLC peaks with a standard curve constructed to relate integrated peak area with known masses of retinol and internal standard retinyl acetate prepared using published extinction coefficients for retinol and retinyl acetate [26]. Retinol was provided by P. Sorter (Hoffmann-La Roche, Nutley, NJ). Retinyl acetate was purchased from Eastman Kodak (Rochester, NY).

Reverse-phase HPLC for assessing serum retinol. Retinol was separated by reverse-phase HPLC on a 250- × 4.6-mm column (Ultrasphere C18 [5 μm]; Beckmann Instruments, Fullerton, CA) using a mobile phase consisting of acetonitrile:methanol:methylene chloride (70:15:15 vol/vol) at a flow rate of 1.8 mL/min. The running column was preceded by a C18 guard column. Retinol was detected at 325 nm with a UV absorbance monitor (996 photo diode array; Waters, Milford, MA).

Induced mutant mice and mouse husbandry. The generation and physiology of RBP-deficient (RBP−/−) mice and RBP-deficient mice overexpressing a transgene for human RBP in skeletal muscle and heart (MCK-hRBP rescue mice) have been described in detail [27] as have the generation and physiology of TTR-deficient (TTR−/−) mouse [28, 29]. RBP−/−, MCK-hRBP rescue mice, TTR−/−, and wild type mice from the same genetic background were maintained ad libitum on a vitamin A–sufficient commercial chow diet (W. F. Fisher & Son, Piscataway NJ). All mice were housed throughout life in a specific virus and pathogen-free barrier environment in accordance with National Institutes of Health guidelines [30].

Western blot analysis for immunoglobulin light and heavy chains. Western blot analysis for mouse IgG light and heavy chains was carried out on mouse plasma samples obtained from RBP−/−, MCK-hRBP rescue, TTR−/−, and wild type mice as described [27]. For this purpose, a rabbit polyclonal antibody against mouse IgG (H+L) was purchased (Santa Cruz, Santa Cruz, CA) and used at a 1:5000 dilution.

ELISA for mouse immunoglobulin light and heavy chains. The alkaline phosphatase–based ELISA was performed with the SBA clonotyping system/AP kit (Southern Biotechnology Associates, Birmingham, AL) by the protocol provided by the supplier. We used goat anti-mouse immunoglobulin (M+G+A) as the capture antibody. For each mouse plasma sample, we used different dilutions (range, 10−3–10−7) of either alkaline phosphatase–labeled goat anti-mouse immunoglobulin, alkaline phosphatase–labeled goat anti-mouse κ chain, or alkaline phosphatase–labeled goat anti-mouse λ chain. The optical density of the samples was read at 405 nm at 10 and 20 min after substrate addition to assure completion of color formation.

FACS analysis of cell types in spleen, thymus, and bone marrow. All analyses were performed on 3-month-old male mice maintained on a control chow diet throughout life. Cells were analyzed on a
Figure 1. Scatter diagram relates serum vitamin A concentration to urinary lactulose/mannitol ratio for 30 mildly malnourished Brazilian children. Regression analysis of data indicates that serum vitamin A concentrations correlate inversely with urine lactulose/mannitol ratios ($R = 0.46, P = .012$). Urine lactulose/mannitol ratio reflects % of each dose excreted in urine. Measurement protocols are described in Methods.

Figure 2. Scatter diagram relates serum vitamin A concentration to urinary mannitol concentration for 30 mildly malnourished Brazilian children. Regression analysis of data indicates that serum vitamin A concentrations correlate directly with urine mannitol concentrations ($R = 0.66, P < .01$). We were unable to detect a statistically significant correlation between serum vitamin A and urine lactulose levels. Since mannitol uptake/excretion is taken as a measure of overall villous surface area, this suggests a role for vitamin A in maintaining the health of the intestinal villous tissue.

Results

Human studies. We explored linkages between serum vitamin A levels and intestinal integrity in pilot studies on 30 apparently healthy children in an impoverished urban neighborhood in Fortaleza, northeast Brazil. Physical examinations were done by a physician or a physician’s assistant. To quantify intestinal barrier disruption, we assessed differential urinary excretion of ingested lactulose and mannitol as respective markers of barrier disruption and overall villous surface area [20–24, 31]. Fasting serum retinol levels were measured to establish the vitamin A nutritional status of the 30 subjects. Figure 1 shows a readily apparent inverse correlation between serum retinol concentration and urinary lactulose/mannitol ratio. Regression analysis showed this relationship to be highly significant ($R = 0.46, P = .012$). Routinely, for comparison purposes, the lactulose/mannitol ratio is considered abnormal or positive if it is $>0.030$ [20–24, 31]. For the 30 children studied, 24 had ratios $>0.030$. Serum retinol levels for the 30 children ranged between 17 and 53 μg/dL serum: 19 children had serum retinol levels of $<30$ μg/dL. Healthy children of the same age in Western Europe or the United States have serum retinol levels of $>30$ μg/dL [8, 9]. Night blindness, a first clinical symptom of VAD, is encountered as serum retinol levels decline to levels approaching 10–15 μg/dL. Thus, for our study, 80% of the subjects (24 of 30) had abnormal lactulose/mannitol test results, yet none could be classified as having clinical or severe VAD [8, 9].

Urinary lactulose and mannitol levels were measured, and the lactulose/mannitol ratio was determined. Only urine mannitol levels correlated with serum retinol levels. A scatter diagram relating serum vitamin A (retinol) level and urine mannitol concentration is shown in figure 2. Regression analysis of these data showed a highly significant correlation between serum vitamin A and urine mannitol levels ($R = 0.66, P < .01$).

Animal studies. To elucidate the biochemical and physiologic mechanisms that underlie linkages between vitamin A nutritional status and immunity, we generated several knockout and transgenic strains of mice. The knockout mice totally lack either RBP or TTR (see [27–29] for additional details). The transgenic mouse model expresses human RBP in skeletal muscle and heart but does not express mouse RBP (MCK-hRBP rescue mice) [27]. The transport and delivery of retinol to tissues is affected in these mice. Table 1 gives the serum retinol levels for wild type, heterozygous RBP-deficient (1 normal copy of the mouse RBP gene), homozygous RBP-deficient, TTR-deficient, and MCK-hRBP rescue mice. Table 1 shows clearly that these mouse strains have very different characteristic circulating...
retinol levels. Both the RBP- and TTR-deficient mice have very low serum retinol levels, similar to those that would be observed in the later stages of VAD. The MCK-hRBP rescue mice have serum retinol levels that are about three-fold higher than those of age- and sex-matched wild type mice. These levels are roughly equivalent to those of healthy adult humans.

Because of interest in vitamin A effects on the immune response, we assessed circulating immunoglobulin levels in age- and sex-matched mice that had been maintained on the same nutritionally complete control diet throughout life. Western blot analyses (data not shown) for immunoglobulin light (k) and heavy chains suggested that circulating immunoglobulin levels in homozygous RBP- and TTR-deficient and MCK-hRBP rescue mice were less than 50% of those of wild type mice. Our Western blot analyses indicated that circulating immunoglobulin levels for heterozygous RBP-deficient mice were intermediate between those of wild type and homozygous RBP-deficient mice.

To quantify immunoglobulin levels of RBP-deficient and MCK-hRBP rescue mice, ELISAs were carried out for total IgG and both the k and λ light chains. These data are presented graphically in figure 3. Indeed, as was suggested by the less quantitative Western blot analyses, total IgG and the k and λ light chain serum concentrations were about 50% lower for RBP-deficient and MCK-hRBP rescue mice than for wild type mice.

We next analyzed different B and T cell populations present in the spleen, thymus, and bone marrow of wild type and RBP- and TTR-deficient mice. By FACS analysis, we screened for cell populations expressing IgM, CD3, CD4, CD8, CD43, and TTR-deficient mice. By FACS analysis, we screened for light chain serum concentrations were about 50% lower for wild type and RBP- and MCK-hRBP rescue mice. Values for 1 representative of 3 experiments are presented. No independent determination varied by >10% from mean shown. y axis gives optical density (O.D.) of samples read at 405 nm 10 min and 20 min after substrate addition to assure completion of color formation. Mice were maintained throughout life ad libitum on a control chow diet. All procedures are described in Methods.

Discussion

We explored how vitamin A nutritional status and intake influence gut integrity. To investigate possible linkages between vitamin A and intestinal integrity, we used the lactulose/mannitol test. This test provides a noninvasive method to assess disrupted intestinal barrier function and etiology-specific effects in disrupting intestinal barrier function. Both lactulose and mannitol are hydrophilic sugars and have low affinity for the monosaccharide transport system. Thus, they are absorbed passively in a carrier-independent fashion [20–23]. After uptake, both sugars are excreted intact into the urine [20–23]. Mannitol, a monosaccharide, has a radius of 0.4 nm and is absorbed transcellularly through aqueous pores in the epithelial cell membrane; hence uptake is proportional to the total epithelial absorptive surface area. In contrast, lactulose, a disaccharide, with a radius of 0.52 nm, is absorbed paracellularly via extrusion zones at the villous tips and at tight junctions. Thus, lactulose uptake is enhanced upon disruption of intestinal barrier function [20–23, 31]. The lactulose/mannitol permeability ratio provides a quantitative measure of intestinal integrity and function. The ratio is increased in celiac and Crohn’s diseases, in diarrhea with malabsorption, and in human immunodeficiency virus–infected patients with and without diarrhea [20–24, 31].

For 30 Brazilian children, serum retinol concentrations showed a significant inverse correlation (R = 0.46, P = .012) with the urinary lactulose/mannitol ratio (see figure 1). Moreover, for this population, serum vitamin A (retinol) concentra-
tions showed a significant direct correlation ($R = 0.66, P < .01$) with urinary mannitol concentrations (see figure 2). Since mannitol uptake was significantly greater in children with higher serum retinol concentrations, this suggests a role for vitamin A in maintaining an intact epithelial absorptive area in the gut. Although it has long been understood that vitamin A is needed to maintain the differentiation state and health of epithelial cells [1, 4, 5], the effect of vitamin A on intestinal barrier function and consequently intestinal health has not been fully appreciated (see note after text).

The serum retinol levels for the 30 children who participated in our study were $\sim 17–53 \mu g/dL$ serum: Levels for most children (27 of 30) clustered at 20–40 $\mu g/dL$. According to World Health Organization guidelines, VAD categories are defined by serum concentrations as follows: <10 $\mu g/dL$, severe; 10–20 $\mu g/dL$, moderate; and 20–30 $\mu g/dL$, mild [8, 9]. By applying these guidelines to our study, 2 subjects would be classified as having moderate VAD and 17 as having mild VAD. Thus, we were able to establish a statistical linkage between serum vitamin A concentrations and intestinal barrier function, as assessed by lactulose/mannitol ratio, for a population of children that would be considered mildly malnourished. On the basis of our data, we predict that persons with severe VAD would be more prone to showing relatively large (abnormal) lactulose/mannitol ratios and impaired intestinal barrier function. Moreover, we propose that, for our patient population, performance in the lactulose/mannitol test would be improved several days after consumption of a dose of supplemental vitamin A. We are now testing these predictions. If our initial findings and predictions can be verified, these data may provide a mechanistic basis to explain why children who can be classified as having subclinical VAD are at increased risk of morbidity and mortality. Nevertheless, our finding of a relationship between serum retinol concentrations and performance in the lactulose/mannitol excretion test represents a correlation and does not specifically address causation.

To study vitamin A effects on the immune response and other essential biologic processes, we generated transgenic and knock-out strains of mice that have markedly different capacities for transport of retinol in the circulation. RBP-deficient and TTR-deficient mice have very low serum retinol levels (see table 1), comparable to those that might be encountered in humans or animals with severe VAD [8, 9, 27–29]. In contrast, a transgenic strain that expresses only human RBP and no mouse RBP (MCK-hRBP rescue mice) have serum retinol levels about three-fold higher than those of wild type mice and comparable to those found in humans. In characterizing the phenotypes of these various mouse strains, we observed that circulating immunoglobulin levels for the RBP- and TTR-deficient and for the MCK-hRBP rescue mice were about half those of age- and sex-matched wild type controls (see figure 3 and Results). Since all of these mice were housed throughout life under the same specific virus and pathogen-free barrier conditions and since all had received the same nutritionally complete chow diet, it is likely that the lower immunoglobulin levels arise from, or are directly linked to, altered retinol transport and/or availability.

We are now initiating studies of the biochemical basis of this immune phenotype and of its physiologic consequences. Although both the RBP- and TTR-deficient mice have very low circulating retinol levels, the biochemical basis for this phenotype is different in the 2 strains. For RBP-deficient mice, hepatic retinol stores cannot be mobilized and the retinol present in the circulation arises directly from recent dietary intake [27]. For TTR-deficient mice, hepatic retinol stores appear to be mobilized normally but, in the absence of TTR, RBP is filtered in the kidney [28, 29]. For TTR-deficient mice, total retinol levels for all extrahepatic tissues examined (spleen, testis, kidney, lung, and heart) were not statistically different from those of wild type mice [29]. Observations based solely on these 2 strains suggest that the low serum retinol levels present in these strains account for diminished immunoglobulin levels. However, unlike the RBP- and TTR-deficient mice, the MCK-hRBP mice have relatively high circulating retinol levels accompanied by immunoglobulin levels (~50%) of those of wild type mice. Thus, circulating retinol concentrations cannot explain the lower serum immunoglobulin levels in these mouse strains. Alternatively, each of these strains has very low or no circulating mouse RBP. It is possible that RBP itself plays a role in maintaining or influencing circulating immunoglobulin levels. However, at present, there is no suggestion of this from the literature.

FACS analysis showed no differences in the relative abundance of various types of B and T cells for spleen, thymus, and bone marrow of wild type and RBP-deficient and TTR-deficient mice. The basis of the lower immunoglobulin levels is, therefore, not understood. Moreover, we do not yet know whether the diminished immunoglobulin levels observed for these mice has physiologic consequences. We are now studying the responses of these mice to viral and bacterial infections and to exposure to substances that modulate the immune response.

Overall, vitamin A plays an important role in maintaining immunity [16, 17]. The actions of vitamin A in maintaining immunity are not focused through one specific mechanism. Instead, vitamin A affects many different aspects of the immune system. Our studies in humans suggest that vitamin A nutritio nal status, as assessed by serum retinol levels, correlates with normal intestinal barrier activity and consequently plays a role in maintaining innate immunity. We believe that this ongoing work illuminates a previously unrecognized action of the vitamin in maintaining optimal human health. It has long been established that vitamin A plays a role in maintaining both cellular and humoral immunity [16, 17]. Our characterization of RBP- and TTR-deficient and MCK-hRBP rescue mice suggests that not only retinol, but RBP as well, may directly affect the immune response.
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References