

Bacterial population and innate immunity-related genes in rat gastrointestinal tract are altered by vitamin A-deficient diet

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Abstract

Vitamin A and its derivatives have been shown to regulate the growth and differentiation of gastrointestinal epithelial cells; in addition, vitamin A deficiency has been convincingly shown to be associated with increased susceptibility to infection. The gastrointestinal mucosal barrier, which is a component of the innate immune system, is considered the first line of defense, as it provides a barrier between the external environment and the internal milieu. A disturbance in the integrity of the intestinal epithelium is one of the main factors involved in increased incidence of infections during vitamin A deficiency. In this study, the effects of vitamin A deficiency on microbial ecology and the expression of genes related to the intestinal mucosa's innate immunity were examined in a rat model. Using the 16s rDNA method, we demonstrate that a vitamin A-deficient (VAD) diet increases the total amount of bacteria in the gastrointestinal tract and alters the intestinal microflora. Results show a decrease in the relative proportion of *Lactobacillus* spp. and the simultaneous appearance of *Escherichia coli* strains. Lack of vitamin A significantly changed mucin (MUC) dynamics, as reflected by the enlarged goblet-cell "cup" area relative to controls; decreased MUC2 mRNA expression in the jejunum, ileum and colon of VAD rats and increased MUC3 mRNA expression in the ileum and colon of these rats. In addition, vitamin A deficiency down-regulated defensin 6 mRNA expression while up-regulating toll-like receptors 2 and 5 mRNA expressions. The current study indicates that vitamin A deficiency interferes with the integrity of the gastrointestinal mucosal barrier. © 2009 Elsevier Inc. All rights reserved.

Keywords: Vitamin A; Mucin; Toll-like receptors; Defensin; Microflora

1. Introduction

Vitamin A and its analogs are essential for normal growth, development, reproduction, cell proliferation, cell differentiation, immune function and vision [1,2].

The integral intestinal epithelium, which depends largely on the continuous proliferation, migration and differentiation of crypt cells [3,4], exhibits rapid cell proliferation and differentiation, making this tissue more susceptible to the effects of vitamin A deficiency (VAD).

In previous studies, we reported that VAD induces morphological and functional changes in chicken and rat small intestines [5–7].

Several studies have shown that vitamin A is of fundamental importance in maintaining the integrity and function of the immune system. Its deficiency is associated with increased susceptibility to infectious diseases in both humans and animal models [8,9].

Epithelial cells are an integral and perhaps the most important part of the innate, natural defense mechanism of mucosal surfaces. The epithelium of the gastrointestinal (GI) tract is covered by mucus, which forms an abundant and continuous layer lining the apical surface of intestinal epithelial cells. This mucosal surface operates a number of protective strategies in defending against pathogens found within the intestinal lumen. Normal mucosal epithelium is permeable only to low-molecular-weight molecules such as

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nutrients and therefore limits the exposure of the intestinal mucosa to potential parasitic, bacterial and viral pathogens in the gut lumen. Pathogenic bacteria are trapped in the mucus as they move down the GI tract. In addition, host commensal floras colonize the adherent mucus layer and, as a result, prevent the attachment of pathogenic organisms [10]. The mucus layer is composed predominantly of mucins (MUCs), which are high-molecular-weight glycoproteins produced and secreted mainly by epithelial goblet cells. They are heavily glycosylated and are strongly charged, as a consequence of sialylation or sulfation [11]. Two types of MUC can be found in the GI tract: the first is a gel-forming MUC, and the second, a membrane-bound one. The major MUCs produced in the rodent intestine are MUC2, produced by the goblet cells, and MUC3. MUC3 has been reported to be expressed in both columnar and goblet cells of the human small intestine and colon [12,13]. The quantity and quality of MUC glycoproteins present in the GI tract are affected by the rates of MUC synthesis and secretion and by the microflora's contribution to MUC degradation. Both pathogenic and commensal bacteria have been reported to be able to enzymatically digest mucus [14]. On the other hand, mucus secretion is typically enhanced in response to intestinal microbes. Both commensal and pathogenic bacteria would derive significant benefit from the ability to regulate mucus synthesis or secretion from host goblet cells [15,16]. Because MUC oligosaccharides provide a source of carbohydrates and peptides, mucus offers numerous ecological advantages to intestinal bacteria [17].

An additional essential factor in maintaining the intestinal epithelial cells' barrier function is the mediation of toll-like receptor (TLR) signaling by commensal bacteria [18]. TLR are membrane-anchored proteins which are variably expressed by different cell types in the intestinal tract. They can recognize different microbial structures and activate a number of innate host defenses. These microbial sensors are type I transmembrane receptors with extracellular leucine-rich repeats and an intracellular signaling domain known as the TIR domain [19,20]. To control bacterial invasion, the host's defense system not only needs to be able to detect their presence: it also needs to be able to control and kill them. The constant production of antimicrobial peptides (e.g., defensin) in the intestinal tract helps limit invasion and adherence of both pathogenic and commensal bacteria [21].

Defensins are small (3–4 kDa), positively charged, arginine-rich microbiocidal peptides with three characteristic pairs of intramolecular disulfide bonds and a β -sheet structure. They cause microbial death by lysis through disruption of the integrity of bacterial membranes, which differ from host-cell membranes in their lack of cholesterol and high content of negatively charged phospholipids. Defensins are divided into two main families, α -defensins and β -defensins, based on the arrangement and spacing of their disulfide bonds [20,22]. Ouellette et al. [23] discovered that α -defensins are expressed in murine Paneth cells.

The aim of the present study was to examine the effect of low vitamin A levels on the rat's GI tract in terms of microbial ecology and innate immunity-related genes.

2. Materials and methods

2.1. Animals

Male Wistar rats with an average weight of 40 g (range, 35–40 g) were obtained from the Harlan Laboratory at The Weizmann Institute of Science, Rehovot, Israel. They were housed in metal cages in a room with controlled temperature ($25\pm 2^\circ\text{C}$), relative humidity ($65\pm 5\%$) and light (0800–2000 h). Ethical approval was obtained for the study. The procedures were conducted in full compliance with the strict guidelines of the Hebrew University Policy on Animal Care and Use.

2.2. Diets

The rats were randomly distributed into VAD ($n=5$) or vitamin A-sufficient (VAS, $n=5$) groups. The VAD group was fed a pelleted version of a previously described VAD diet [24] (cat. #960220, ICN Nutritional Biochemicals, Costa Mesa, CA, USA) (Table 1). The VAS rats were fed a custom control diet for vitamin A deficiency containing retinyl palmitate (1200- $\mu\text{g}/\text{kg}$ diet; 3000-IU/kg diet) (ICN Nutritional Biochemicals) and were pair-fed with the VAD rats.

Both group of rats had free access to water. The diets contained (on a dry weight basis) 5% fat, 20% protein, 60% carbohydrate and optimal amounts of the other essential nutrients. Food intake was monitored daily, and the rats were weighed every other day to compare the growth curve plateaus in the VAD versus VAS group as a measure of the VAD state.

2.3. Samples collection

After 7 weeks of feeding, when the VAD rats reached a plateau in growth, the rats were sacrificed by cervical

Table 1
Composition of VAD diet^a

Ingredients	Quantity (g/kg)
Vitamin-free casein	180
DL-methionine	3
Cornstarch	298.5
Sucrose	298.5
Alphacel, nonnutritive bulk	50
Cottonseed oil	50
Brewers yeast	80
Vioosterol	0.011
Minerals ^b	6.53
Salt mixture No. 2, USP XIII ^c	40

^a Catalogue no: 960220, ICN Biomedicals, Costa Mesa, CA, USA.

^b Minerals (g/kg diet): calcium carbonate, 6.250; manganese sulfate $\cdot\text{H}_2\text{O}$, 0.180; zinc carbonate, 0.050; cupric sulfate $\cdot 5\text{H}_2\text{O}$, 0.025; chromium potassium sulfate, 0.022; sodium fluoride, 0.005; potassium iodide, 0.001; sodium selenite, 0.001.

^c Components (g/kg diet): calcium biphosphate, 5.43; calcium lactate, 13.08; ferric citrate (16–17% Fe), 1.18; magnesium sulfate, 5.48; potassium phosphate dibasic, 9.60; sodium biphosphate, 3.49; sodium chloride, 1.74.

dislocation. Blood was collected from the portal vein. Tissue samples were taken from the jejunum, ileum, colon and liver. Samples for mRNA determination were snap-frozen in liquid nitrogen and stored at 80°C until analysis. Tissue samples were taken for histology, washed in phosphate-buffered saline (PBS) and fixed in 4% (v/v) buffered formalin. Samples for microbial identification were taken from the jejunum, ileum, colon and feces and stored at -20°C .

2.4. Retinol determination

Liver and plasma vitamin A were extracted from liver and plasma samples, respectively. For serum retinol determinations, 200 μl of serum were deproteinized with 200 μl ethanol [containing 0.4 μg retinyl acetate (Sigma Chemicals, St. Louis, MO, USA) as an internal standard]. Then 1 ml petroleum ether was added, and the mixture was vortexed twice and centrifuged for 10 min at 1000g. The upper phase was collected, dried under nitrogen and reconstituted with 100 μl methanol. Retinol was assayed by reverse-phase high-performance liquid chromatography (HPLC) (Merck C18, 5 μm , 4.6 \times 150 mm, Merck, Darmstadt, Germany), using methanol/acetic acid (99:1, v/v) as the mobile phase, and ultraviolet detection (multiwavelength detector model Md-910, Jasco, Tokyo, Japan) at 325 nm [25]. For tissue analysis of retinol contents, 3 ml ethanol and 0.75 ml KOH (50% in water) were added to 1 g of sample, stirred and kept at 60°C for 30 min. The samples were cooled to room temperature and extracted three times in 3 ml petroleum ether and 2 ml deionized water. The upper phase was collected, and 2 ml of 1% HCl in water and 10 μg retinyl acetate (Sigma Chemicals), as an internal standard, were added. The solvent was evaporated under N_2 , and the sample was dissolved in 1 ml of methanol. HPLC analysis was as described for serum retinol.

2.5. Histological examination

Fresh sections of colon, ileum and jejunum tissue were fixed overnight in 4% (v/v) buffered formaldehyde. Serial 5- μm sections were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in chloroform and embedded in paraffin. For morphometric analysis, sections were deparaffinized in xylene, rehydrated, stained with hematoxylin and eosin and evaluated by light microscopy.

2.6. Morphometric measurements

The goblet-cell area was calculated from the length and width of the goblet cell cup in cross sections of the villi as previously described [26]. Measurements were performed with an Olympus light microscope using EPIX XCAP software.

2.7. Bacterial genomic DNA extraction

Bacterial genomic DNA was isolated from tissues using a Wizard Genomic DNA purification kit according to the manufacturer's protocol (Promega, Madison, WI, USA).

Feces were treated as previously described by Zhu et al. [27]. They were homogenized by vortexing with 4-mm diameter glass beads for 3 min; debris was removed by centrifugation at 700g for 1 min; the supernatant was collected and centrifuged at 12,000g for 5 min. The pellet was washed twice with PBS and stored at -20°C until DNA extraction [27–29]. For DNA purification, the pellet was resuspended in EDTA and treated with 10 g/L lysozyme (Sigma Aldrich, St. Louis, MO, USA) for 45 min at 37°C . The bacterial genomic DNA was then isolated, and its concentration and purity were checked spectrophotometrically.

2.8. Polymerase chain reaction amplification of bacterial 16s rDNA

Primers for *Lactobacillus* spp. were designed according to Wang et al. [30], the *Bifidobacterium* spp. primer set was as described by Langendijk et al. [31] and the *E. coli* strain primer set was designed according to Tsen et al. [32]. Universal primers identifying all known bacteria were designed according to Amit-Romach et al. [28]. The universal primer set was used to determine the total microflora population [31,33]. The primers used to amplify bacterial 16s rDNA are shown in Table 2.

For polymerase chain reaction (PCR) amplification of the bacterial targets from tissue, 100 ng of extracted DNA was amplified and for the bacterial targets from feces, 50 ng of extracted DNA was amplified using GoTaq Flexi DNA polymerase (Promega).

DNA (5 μl) was added to 45 μl of PCR mixture containing 19.5 μl of nuclease-free water, 5 μl of each

Table 2
PCR primers used to amplify bacterial 16s rDNA

Bacterial group	Primers	Sequence (5'-3')	Length (bp)
Universal	Unibac-f	CGTGCCAGCCGCGGTAATACG	611
	Unibac-r	GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT	
<i>Lactobacillus</i> spp.	LAA-f	CATCCAGTGCAAACCTAAGAG	286
	LAA-r	GATCCGTTGCGCTTCGCA	
<i>E. coli</i> strain	<i>E. coli</i> -f	GGGAGTAAAGTTAATACCTTTGCTC	584
	<i>E. coli</i> -r	TTCCCGAAGGCACATTCT	
<i>Bifidobacterium</i> spp.	Bif164-f	GGGTGGTAATGCCGGATG	510
	Bif662-r	CCACCGTTACACCGGGAA	

primer, 1 μ l of nucleotide (deoxyribonucleotide triphosphate) mix, 10 μ l of PCR buffer, 4 μ l of MgCl₂ and 0.5 μ l of Taq polymerase. To determine the linear phase of the amplification, the PCR was run with different numbers of cycles (25, 30, 35, 40, 45 or 50) for each primer set. Amplification of the fragments was as follows: *Lactobacillus* spp., a 286-bp 16S rDNA fragment, 35 cycles; *Bifidobacterium* spp., a 510-bp 16S rDNA fragment, 42 cycles; *E. coli* strains, a 584-bp 16S rDNA fragment, 42 cycles; universal primers, a 611-bp 16S rDNA PCR product, 37 cycles.

Amplification conditions were denaturation (94.5°C, 30 s), annealing (60°C, 1 min) and extension (72°C, 50 s).

PCR products were visualized by agarose gel (2%) electrophoresis stained with ethidium bromide and quantified with a Gel-Pro Analyzer version 3.0 (Media Cybernetics, Bethesda, MD, USA). The evaluation of the different PCR products was normalized to the density of the PCR product of the universal primers by densitometer scanning and was reported in arbitrary units as described previously [28].

2.9. Total RNA isolation

Total RNA was isolated from the intestinal segments using TRI-Reagent-RNA/DNA/protein isolation reagent 5 (1 ml/100 mg of tissue) according to the manufacturer's protocol (Sigma Chemical). The integrity of the RNA was verified by ethidium-bromide staining, and its concentration was determined spectrophotometrically.

2.10. mRNA analysis

Reverse transcriptase-PCR (RT-PCR) was carried out with primers for MUCs, TLR and defensin. MUC mRNA expression was examined using RT-PCR with primers for two types of MUC: MUC2 and MUC3. The primers were designed according to Amit-Romach et al. [34].

TLR genes were tested with primers for TLR2 and TLR5. TLR2 primers were designed from the fragment of *Rattus norvegicus* TLR2 mRNA (GI 42476288), and TLR5 primers from the predicted *R. norvegicus* TLR5 mRNA (GI 109499200).

To examine defensin mRNA expression, primers for defensin 6 were designed from the fragment of *R. norvegicus* defensin 6 gene, complete cds (GI 50981008).

β -Actin was used as a housekeeping gene and designed according to Amit-Romach et al. [34]. The primers used for RT-PCR are shown in Table 3.

Total RNA was amplified using the Promega Access RT-PCR System with the following program: 30 s at 94°C, 1 min at 60°C, 30 s at 68°C for 35 cycles followed by 7 min at 68°C. The RT-PCR products were examined on a 2% agarose gel and visualized by staining with ethidium bromide.

The PCR was run with different numbers of cycles (25, 30, 35, 40, 45 or 50) for each primer set in order to find the center of the exponential increase in PCR product.

Table 3
PCR primers used to amplify mRNA

Genes	Primers	Sequence (5'-3')	Length (bp)
MUC2	MUC2-f	CAGAGTGCATCAGTGGCTGT	242
	MUC2-r	CCCCTCGAAGGTGATGTAGT	
MUC3	MUC3-f	AACTGCGACTGGGGCACCCAGAAA	366
	MUC3-r	AAAACCGTTTTTGTGTTAGTAT	
TLR2	TLR2-f	ATCTCAGAGGATGCTGCAAGC	648
	TLR2-r	AGGAAAGCAGACTCGTCAA	
TLR5	TLR5-f	ATGGCGTATCAGCTTGACCTT	475
	TLR5-r	TGTGAATCTCGTTGGCAGAG	
Defensin 6	DEF6-f	ACATCAGGATGGAAGCCTTG	376
	DEF6-r	GCACTCACCTGCATCTTGAA	
β -Actin	β -Actin-f	AACTGGGACGATATGGAGAAGATTT	252
	β -Actin-r	TGGGCACAGTGTGGG TGA	

2.11. Statistical analysis

Values are presented as means \pm S.D. The effects of vitamin A status were analyzed by Student's *t* test, carried out within segments but not between them. A post hoc test (Tukey–Kramer) was performed when the interaction between treatments was significant. Differences were considered significant at $P < .05$. JMP version 5.1 [35] was used for all analyses.

3. Results

3.1. Food consumption and weight gain

There was no difference in average daily food intake among the groups throughout the experiment (data not shown). On Days 35–42, VAD rats started to gain less body weight than they had earlier. On Days 48–51, the body weight of VAD rats began to plateau, whereas the VAS rats continued to gain weight. Mean body weight was significantly less ($P < .05$) in the VAD group than in the VAS group from Day 43 on. On days 52–54, the final mean body weight of VAD rats (345 \pm 4.26 g) was significantly lower ($P < .05$) than that of VAS rats (395.2 \pm 9.2 g).

3.2. Liver and serum vitamin A

Consumption of a VAD diet for 7 weeks led to a marked reduction (by 75% and 99%, respectively) in serum and liver retinol relative to the control VAS rats ($P < .001$). Serum concentrations was 15.19 \pm 1.19 (μ g/dl) in rats fed VAD and 62.34 \pm 3.75 (μ g/dl) in VAS diets. At the same time, liver retinol concentrations in rats fed VAD was 0.93 \pm 0.31 (μ g/g tissue) and 117.82 \pm 4.96 (μ g/g tissue) in VAS diets.

3.3. Bacterial populations

Analysis of the microbial ecology of the different intestinal segments showed that feeding a VAD diet increases the overall amount of bacteria in all examined intestinal segments (Fig. 1).

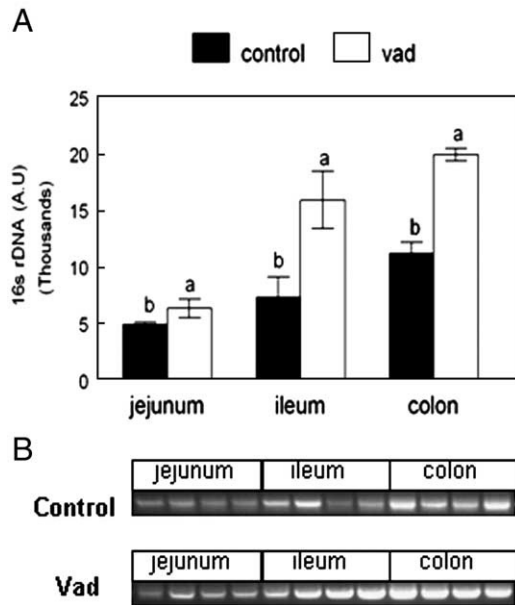


Fig. 1. Intestinal microflora in VAD and control (VAS) rats. (A) The overall amount of bacteria along the GI tract. (B) Representative PCR products of 16S rDNA invariant sequences of all known intestinal bacterial species (universal) in the intestine of VAD and control rats. Values are means±S.E.M., $n=5$. Intestinal segment means without a common letter differ ($P<.05$).

The results indicated that the relative amounts *Lactobacillus* spp. decreased in VAD rats by 62% in the jejunum, 82% in the ileum and 86% in the colon in compare to controls ($P<.05$). Nevertheless, *Bifidobacterium* spp. were not detectable in the examined segments. In addition, vitamin A deficiency led to the appearance of *E. coli* strains in the rat's intestine, particularly in the colon. In contrast, the relative amounts of *Lactobacillus* and *Bifidobacterium* spp. in the feces were not affected by the diet.

3.4. Morphological changes

Vitamin A deficiency led to morphological changes in the ileum and colon. Morphometric measurements of the goblet-cell cup area revealed that VAD enlarged this area in the ileum by 34%, and there was a change in their scattering along the colon: in the control colon, goblet cells were present mainly in the lower part of the crypts, while in the VAD colon, goblet cells were scattered throughout the entire crypt.

3.5. MUC gene expression

A comparison of MUC mRNA expression relative to that of β -actin showed a decrease in MUC2 mRNA expression in the VAD group compared to the controls: by 83% in the jejunum, 56% in the ileum and 75% in the colon ($P<.05$ for all segments) (Fig. 2). In contrast, vitamin A deficiency increased the expression of MUC3 mRNA in the ileum and colon relative to controls, whereas in the jejunum, this

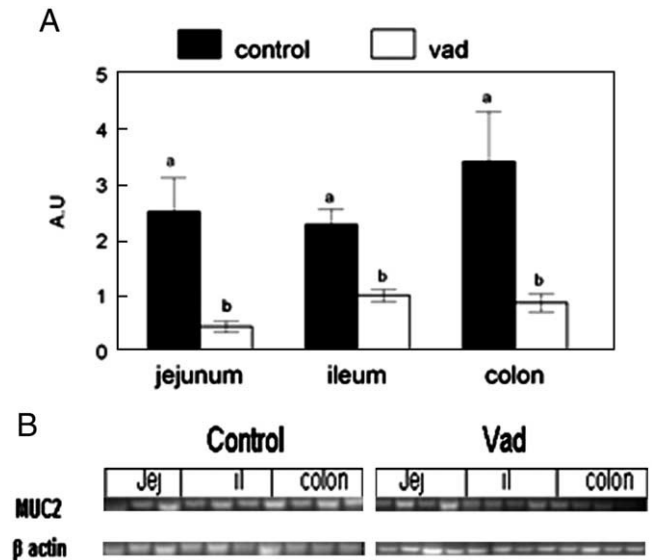


Fig. 2. The effect of vitamin A deficiency on rat intestinal MUC2 mRNA expression. (A) Changes in MUC2 mRNA expression along the intestine were measured by semiquantitative RT-PCR, and β -actin was used as a housekeeping gene. (B) Representative RT-PCR products of intestinal MUC2 and β -actin mRNA expression in the GI tract of control and VAD rats. Values are means±S.E.M., $n=5$. Means without a common letter differ ($P<.05$).

expression decreased. There was a 29% decrease in the jejunum, an 83% increase in the ileum and a 53% increase in the colon relative to controls ($P<.05$).

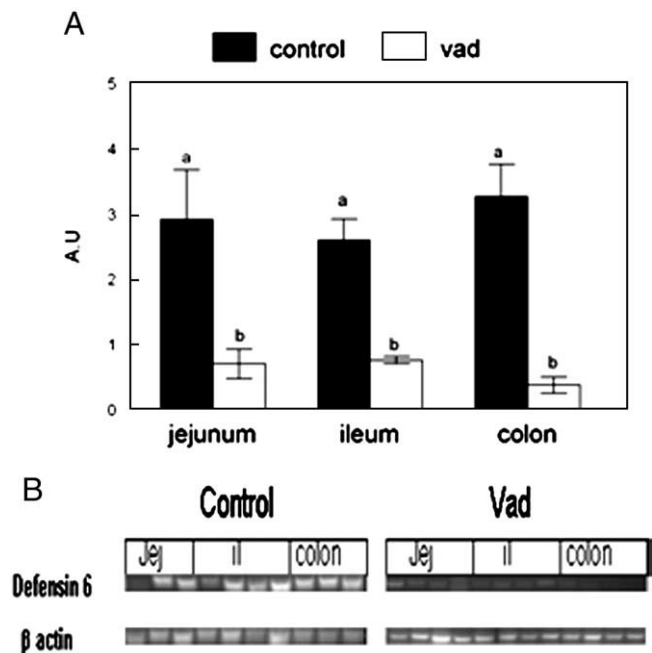


Fig. 3. The effect of vitamin A deficiency on defensin 6 mRNA. (A) Changes in defensin 6 mRNA expression along the intestine were measured by semiquantitative RT-PCR, and β -actin was used as a housekeeping gene. (B) Representative RT-PCR products of intestinal defensin 6 and β -actin mRNA expression in the GI tract of control and VAD rats. Values are means±S.E.M., $n=5$. Means without a common letter differ ($P<.05$).

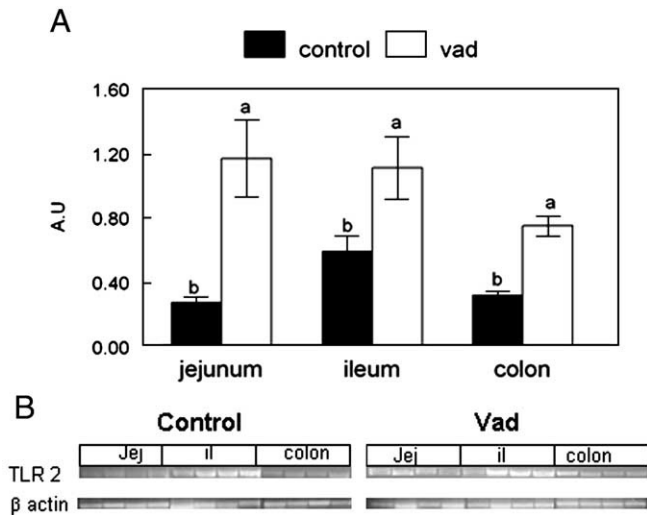


Fig. 4. The effect of vitamin A deficiency on TLR2 mRNA. (A) Changes in TLR2 mRNA expression along the intestine were measured by semi-quantitative RT-PCR, and β -actin was used as a housekeeping gene. (B) Representative RT-PCR products of intestinal TLR2 and β -actin mRNA expression in the GI tract of control and VAD rats. Values are means \pm S.E. M., $n=5$. Means without a common letter differ ($P<0.05$).

3.6. Defensin 6 expression in the jejunum, ileum and colon

A marked decrease in defensin 6 mRNA expression was detected in the VAD intestine. Expression of defensin 6 mRNA decreased by 76% in the jejunum, 71% in the ileum and 89% in the colon, compared to controls ($P<0.05$) (Fig. 3).

3.7. TLR mRNA expression

Vitamin A deficiency up-regulated TLR2 mRNA expression in all three segments (jejunum, ileum and colon). TLR2 mRNA levels were enhanced by 326% in the jejunum, 88% in the ileum and 135% in the colon relative to controls ($P<0.05$) (Fig. 4).

TLR5 mRNA level was elevated in the VAD ileum and colon relative to controls, by 134 and 141%, respectively ($P<0.05$). In the jejunum, on the other hand, TLR5 mRNA levels tended to increase, albeit nonsignificantly.

4. Discussion

The primary goal of this study was to evaluate the effects of VAD on bacterial population and innate immunity-related genes in the rat intestine.

VAD was induced in rats by feeding them a diet deficient in vitamin A for 50–54 days. Consistent with our previous findings in rats and chicks [5,6], no overt clinical symptoms were observed other than the body weight plateau. Deficiency state in the experimental animals was confirmed by the demonstration of reduced concentrations of both hepatic and serum vitamin A.

The present study indicates that VAD changes the production of MUC and in turn its accumulation in goblet cells: goblet cell size was enhanced in the small intestine, these cells' dispersion pattern along the colon changed, and MUC mRNA expression was altered. Lack of vitamin A led to a decrease in MUC2 expression throughout the rat intestine, concurrent with a decrease in MUC3 expression in the jejunum and its increase in the ileum and colon. The most interesting part of these findings was the dissimilar responses of the different parts of the intestine to VAD. MUC2 gene is expressed solely in goblet cells; whereas gene-encoding MUC3 is expressed in both goblet cells and enterocytes [12]. Damage to goblet cells secreting mechanism may occur and cause a reduction in MUC2, which is a gel-forming MUC type, expression. Changes in MUC3 expression may be caused by altered MUCs type. In addition, studies have reported that MUC3 encodes both an apical membrane-associated form and a soluble nonmembrane form of MUC due to alternative splicing [36] and, so, will not be affected by goblet cells-secreting mechanism.

Other works have also demonstrated the effect of vitamin A deficiency on MUC gene expression. Manna et al. [37] found MUC expression in rat tracheal organ culture to be barely detectable in the absence of retinoic acid. Changes in MUC expression may be explained by the ability of vitamin A and related compounds to regulate cell proliferation, differentiation and morphogenesis [38], as well as its ability to modulate gene expression. Guzman et al. [39] have demonstrated that the level of MUC2 and MUC5AC mRNA in cultures of normal human tracheobronchial epithelia is increased by retinoids. Other studies have indicated that the major retinoid receptor mediating the induction of MUC gene expression by retinoic acid (RA) is RA receptor α (RAR α) [40]. RARs have been shown to regulate the expression of a variety of growth factors involved in cellular differentiation [41].

We examined the effect of vitamin A on defensin 6 mRNA expression in rats. The results indicate that vitamin A deficiency caused a decline in the expression of defensin 6 in all intestinal segments examined. This finding may be related to the potential role of vitamin A and its analogs in intestinal epithelial cell proliferation and differentiation [38]. Nevertheless, the results may be explained by morphological alterations of Paneth cells during vitamin A deficiency [42].

The recognition of commensal and pathogenic bacteria by gut epithelia is mediated by pattern-recognition receptors (PRR), also called pattern-recognition molecules [43]. In the gut, TLR play a crucial role in pathogen recognition by the mucosal immune system [44].

Expression of two types of TLR (TLR2 and TLR5) was examined. TLR2 is a receptor for Gram-positive peptidoglycan and lipopeptides [45], and TLR5 recognizes bacterial flagellin, the principal structural component of bacterial flagella [46]. Up-regulation of both TLR2 and TLR5 mRNA expression was displayed throughout the VAD rat intestine. These results are consistent with other reports: recent studies have indicated that TLR2 is present in only small amounts on

epithelial cells in the intestine; hence, there is minimal recognition of luminal bacteria in normal, healthy intestines [47]. Gewirtz et al. [48] reported that TLR5 is localized in the basolateral membrane of intestinal epithelial cells, thus representing a PRR for motile pathogens.

Our findings lead us to conclude that VAD compromises the GI mucosal epithelial barrier's function and, in turn, to impaired mucosal immune responses [49]. The inability of VAD mucosal epithelia to regenerate sufficiently may allow easier penetration of the gut mucosal barrier by potentially pathogenic bacteria. One of the main changes caused by VAD is the diminished number of mucus-producing goblet cells. This loss of the protective mucus blanket, the host's first line of defense, disrupts the barrier between the mucosal epithelium and the lumen of the GI tract and its contents [2]. Once the mucus barrier is breached, invasion occurs: the invading pathogens gain access to the intestinal epithelium and reach the host's cellular surface. At these sites, only microbes that can adhere to mucosal surfaces are able to remain there [50]. In this study, we examined only mRNA expression, and the results cannot be translated to protein amount or activity.

Our results indicate that a lack of vitamin A enables bacterial overgrowth in the rat intestine. This confirms the findings of others who used traditional culturing methods [51]. In addition, alterations in the intestinal microflora were displayed in our study. We demonstrated that VAD leads to a reduction in the proportion of *Lactobacillus* spp. in the GI tract; it also resulted in the appearance of pathogenic *E. coli* strains [32]. Consistent with our present findings, others have reported that MUCs isolated from the intestinal tracts of animals inhibit in vitro adherence of both animal and human enteropathogenic *E. coli* strains [16,52].

In this study, the microbial profile of the mucosa-associated bacteria was found to differ from that of feces-associated bacteria. Our observations are in accordance with Zoetendal et al. [53], who used DGGE (Denaturing Gradient Gel Electrophoresis) analysis of 16S rDNA amplicons and reported that the human GI tract segments are highly similar to each other, while they differ from those of fecal samples. Binding to epithelial cells is the first step for many enteric pathogens in affecting the host, and therefore, feces may contain mainly those bacteria which could not adhere. As such, they do not necessarily provide an accurate reflection of the intestinal microbial ecology. Because we isolated the strains from the intestinal tissue as well as from the feces, these may better reflect the actual colonized intestinal microbiota.

In summary, the results indicate that VAD may cause disruption of the GI mucosal barrier, i.e., the system comprising the immunological network termed gut-associated lymphoid tissue [54].

We suggest that VAD affects the innate immunity and microbial ecology in rat intestine via a multistep mechanism (Fig. 5). VAD modulates cell proliferation and differentiation, which leads to immature epithelial cells, among them Paneth cells. Immature, unlike mature, Paneth cells cannot fully express defensins. The reduction in defensin expression allows

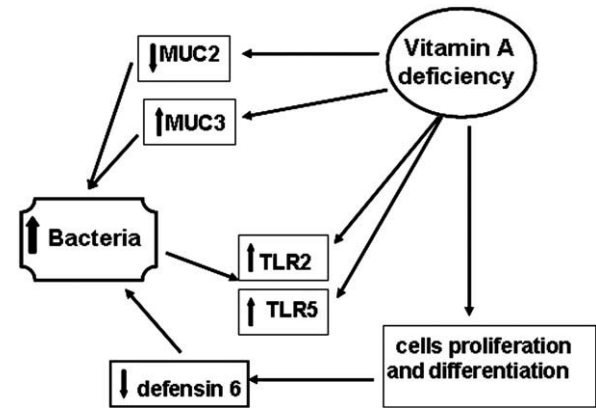


Fig. 5. The proposed schematic illustration for the multistep mechanism effect of vitamin A deficiency on the innate immune system and microbial ecology in rat intestine. Lack of vitamin A leads to a decrease in MUC and defensin expression; this causes a general overgrowth of bacteria. The presence of bacteria at the cellular surface activates TLR reaction.

penetration of pathogenic bacteria through the host's cellular surface and a general overgrowth of bacteria. Furthermore, lack of vitamin A leads to a decrease in MUC expression, which plays a fundamental role in the host's first line of defense. This, in turn, disrupts the barrier between the mucosal epithelium and the GI tract luminal contents, and enables adherence of pathogenic bacteria to intestinal epithelial cells. The invasion of microbes, both pathogenic and opportunistic commensal, activates the TLR response. Our findings may have relevance to the overall vitamin A syndrome and may shed light on the mechanism by which vitamin A deficiency exerts its action to undermine the immune system leading to vulnerability to gastrointestinal morbidity.

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