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Increased Translocation of *Escherichia coli* and Development of Arthritis in Vitamin A-Deficient Rats

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We studied the immune response and the colonization pattern in vitamin A-deficient rats that were colonized with the *Escherichia coli* O6 K13 pOmp 21 strain, which is genetically manipulated to produce ovalbumin and to be resistant to ampicillin. In the vitamin A-deficient rats, the number of bacteria per gram of feces was about five times higher than in the paired fed control rats 4 weeks after colonization. In the control rats, the colon and the lower part of the ileum were colonized, while in the vitamin A-deficient rats all parts of the small intestine, as well as the colon, were heavily inhabited by bacteria. Furthermore, in 75% of the vitamin A-deficient rats, the *E. coli* bacteria were found in the mesenteric lymph nodes, and in 50% of the rats *E. coli* were found in the kidneys. These animals also developed severe arthritis. The levels of serum immunoglobulin G (IgG), IgM, IgE, and biliary IgA antibodies against the bacterial antigens were significantly higher in the vitamin A-deficient rats than in the control rats. The number of IgA-producing cells in the lamina propria of the small intestine was significantly lower in the vitamin A-deficient rats than in the control rats; however, there was an increase in the number of $CD8^+$ cells and transforming growth factor β-producing cells in the lamina propria of the vitamin A-deficient rats. Disturbances in T-cell function were demonstrated, since spleen cells from the vitamin A-deficient rats produced more gamma interferon and interleukin-2 in vitro than control spleen cells. In summary, vitamin A deficiency led to a decrease in the ability to control the localization of intestinal bacteria and an increase in translocation, which was followed by development of arthritis regardless of substantial levels of antibacterial antibodies. The bacterial invasion made the animals hyperresponsive to the bacterial antigens, despite the fact that vitamin A deficiency is normally associated with suppressed antibody production, as previously shown by us and others.

Vitamin A deficiency is one of the major nutritional deficiency syndromes in developing countries and is associated with an increased incidence of infectious diseases. Among these, diarrheal diseases are the main causes of increased child mortality (6, 42). Clinical studies have shown that supplementation with vitamin A decreases the severity of the diseases and the mortality rates (8, 23, 41, 45). Accordingly, it has been shown that vitamin A deficiency leads to a down regulation of effector functions of the immune system, such as impaired antibody production in response to bacterial and viral antigens and depressed T-cell responses in vivo and in vitro (10, 20, 30, 34, 37, 39). In rats, we previously found that vitamin A deficiency results in a diminished ability to respond to a perorally given cholera vaccine, which is manifested as low levels of immunoglobulin A (IgA) anti-cholera toxin antibodies in bile, as well as small numbers of IgA antibody-producing cells in the mesenteric lymph nodes (MLN) (47).

From experimental studies, it is also known that vitamin A deficiency causes alterations in the growth and differentiation of epithelial cells and decreases the number of mucus-producing goblet cells in the gut (35). These changes cause an increase in the vulnerability of the intestinal mucosa (2, 49) and thus might lead to a higher risk for infections because of translocation of intestinal bacteria. Translocation of bacteria is defined as the presence of intestinal bacteria in extraintestinal organs, and it is a function of the integrity of the intestinal epithelium, the number of bacteria in the intestinal tract, and the status of the host immune system (5). The role of the immune system is illustrated by the finding that T-cell deficiency results in an increased translocation without any apparent changes of the permeability of the intestinal epithelium (21). We have previously shown that T cells from vitamin A-deficient rats have a dysfunction and are hyperresponsive in vitro and produce more interleukin-2 (IL-2) and gamma interferon (IFN-γ) than T cells from normal animals (48).

Thus, vitamin A deficiency causes disturbances such as changes in the integrity of the intestinal epithelium, decreased levels of intestinal IgA antibodies, and aberrations in T-cell functions, all of which are factors reported to influence translocation of bacteria.

Therefore, we wanted to study the influence of vitamin A deficiency on the intestinal flora and the translocation as well as the immune response against the intestinal bacteria by colonizing rats with an ampicillin-resistant *Escherichia coli* strain that was genetically manipulated to produce ovalbumin.

MATERIALS AND METHODS

Animals and diet design. Weanling male Wistar rats (60 g) were obtained from BK Universal, Stockholm, Sweden. They were divided into two groups of 12 rats each and were fed either a vitamin A-deficient diet (AnalyCen, Lidköping, Sweden) ad libitum or a control diet as previously described (47). The food consumption of the vitamin A-deficient animals was estimated daily, and the control rats were given the same amount of the control diet as the amount of the vitamin A-deficient diet consumed by deficient rats. All animals were weighed twice a week. The experiment was started 4 weeks after onset of the diet when the animals had reached a weight plateau as a first clinical sign of vitamin A deficiency.

Colonization and immunization. Before colonization with the bacteria, the animals were pretreated with antibiotics for 2 days to reduce the competition from the normal flora of the rats. Streptomycin (5 g/liter; Evans Med. Ltd., Langhurst, Horsham, United Kingdom) was added in the drinking water for 1

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day, and ampicillin (0.5 g/liter; doktacillin; Astra, Södertälje, Sweden) was added for another day. Rectal samples were taken and cultured on Drigalsky and tryptic soy agar plates containing ampicillin to confirm that the antibiotic treatment was sufficient. The rats were starved for 24 h and then tube fed 10^{10} CFU of *E. coli* O6 K13 pOmp 21 in 1 ml of bicarbonate buffer (0.2 M). This *E. coli* strain has been genetically manipulated to produce ovalbumin (OA) by introduction of the plasmid pOmp 21, which also carries a gene that makes the bacteria resistant to ampicillin and streptomycin (12, 32, 33). The rats were given ampicillin containing water during the whole experimental period to prevent loss of the plasmid by the bacteria. Fecal cultures were made on Drigalski plates and tryptic soy agar plates containing ampicillin the day after colonization and weekly thereafter. Three weeks after colonization, the rats were immunized subcutaneously with 10^9 CFU of formalin-killed *E. coli* of the wild type in 1 ml of phosphate-buffered saline (PBS) and were sacrificed 7 days thereafter.

Sample collection. Blood samples were taken before colonization and before and 7 days after immunization, and serum was prepared. Bile was collected at day 28 after colonization while the rats were anesthetized with pentobarbital (6 mg/100 g of body weight intraperitoneally; Mebumal; Nord Vacc, Piteå, Sweden) by insertion of a plastic tube (PE 20 Intramedic; Clay Adams, Parsippany, N.J.) into the bile duct (11). Serum and bile samples were stored at −20°C until they were analyzed for immunoglobulin and antibody levels.

Liver biopsy samples were taken and used, together with the serum samples, for determination of retinol levels according to the method described by Catignani et al. (9, 25).

Spleens were removed aseptically, and cell suspensions were prepared. The organs were minced and filtered through sterile nylon filters (Becton Dickinson). The cells were washed and resuspended in complete medium (Iscoves, 10% fetal calf serum [FCS], 1% L-glutamine, 1% gentamicin) to the proper concentration.

Bacterial cultures. Bacterial cultures from the blood, kidneys, MLN, Peyer's patches, and different parts of the intestine were taken from animals that were killed on days 1, 3, 7, 11, and 28 after colonization.

Blood was obtained by heart puncturing, and 0.1-ml samples were cultured on 5% horse blood agar plates.

Kidneys were removed aseptically and divided with a sterile scalpel, and impression cultures of the exposed surface were done on 5% horse blood agar plates.

MLN were minced individually and grown overnight in brain heart infusion broth containing 0.1% ampicillin. Thereafter, 0.1 ml of the broth was transferred onto tryptic soy agar plates containing ampicillin.

Bacterial cultures from the Peyer's patches were taken by making an incision in the serosal wall of the patch and pressing a sterile loop on the exposed interior, with careful avoidance of contamination from the gut lumen. The loop contents were cultured on Drigalski plates and tryptic soy agar plates containing ampicillin.

Samples from the duodenum, jejunum, and ileum mucus layers were taken with sterile loops and cultured onto Drigalski plates and ampicillin-containing tryptic soy agar plates.

To determine the amount of bacteria per gram of feces in the cecum, 10-μl feces samples were collected with sterile loops and diluted in 10 ml of sterile PBS. The samples, which were diluted 10^{-3}, 10^{-5}, and 10^{-7}, were cultured on Drigalsky plates.

Arthritic limbs, which were defined by swelling and erythema of the joints, were observed 28 days after colonization. Samples were taken after dissection of the talucrural joint of the hind paw and/or the knee with charcoaled sticks that were pressed against the opened joint, and the sticks were pressed onto 5% horse blood agar plates. For animals that did not develop arthritis, cultures were taken from the left knee.

Typing of the bacteria. The *E. coli* bacteria were tested for type 1 fimbrial expression by agglutination of guinea pig erythrocytes in a mannose-reversible manner (16, 17). Serotyping of the O6 lipopolysaccharide (LPS) and the K13 capsular polysaccharide was performed as described elsewhere (35a). The *E. coli* cells were cultured in brain heart infusion broth overnight, and the presence of OA in the supernatants was determined by an enzyme-linked immunosorbent assay (ELISA) technique as described below.

Sample collection for immunohistochemistry and histology. One-centimeter-long pieces from the jejunum, with or without Peyer's patches, were fixed in periodate lysine paraformaldehyde (PLP) for 16 to 24 h at 4°C. The PLP fixative contained lysine phosphate buffer (0.2 M lysine monohydrochloride, 0.1 M sodium dihydrophosphate [pH 7.4]) and 8% paraformaldehyde (paraformaldehyde, 10 M NaOH, 5.4 g of glucose), which were mixed in a ratio of 3:1 just before use. Solid sodium metaperiodate was added to the mixture at a final concentration of 0.01 M. The PLP-fixed gut pieces were embedded in paraffin and stored at −20°C until sections were taken.

Joints were fixed in formaldehyde for at least 3 weeks and were then used for histopathological analysis.

Immunohistochemistry. Five-micrometer-thick sections were cut from the PLP-fixed gut biopsies and fixed in xylene and alcohol. Monoclonal anti-rat IgA (1/500; Serotec, Oxford, England), monoclonal anti-rat CD8 (1/500; Serotec), and monoclonal anti-transforming growth factor β (TGF-β) antibodies (1/20; Genzyme, Cambridge, Mass.) were applied and incubated for 1 h. Biotinylated anti-mouse IgG antibodies (1/500; Serotec) were applied for another hour. Streptavidin-biotin complex (Dako A/S) conjugated to horseradish peroxidase was incubated for 30 min. The sections were finally treated for 10 min with the substrate solution (10 mg of aminoethylcarbazole, 6 ml of dimethylsulfoxide, 50 ml of 0.02 M sodium acetate buffer [pH 5.5], 10 μl of 30% H_2O_2), washed in tap water, and counter stained for 5 min with Mayer's hematoxylin.

To estimate the numbers of IgA^+, $CD8^+$, and TGF-β^+ cells, a computer-assisted image analyzer (MicroScale TM/TC Image Analyser System; Digithurst Ltd., Royston, England) was used. Five defined areas per section were randomly chosen, and the surface areas of the stained cells within these areas were measured. The relative areas expressing IgA^+, $CD8^+$ and TGF-β^+ cells were calculated as the area of stained cells over the total area of the five defined areas.

Determination of immunoglobulin concentrations. Microtiter plates (round-bottom MIC-2000; Dynatech, Alexandria, Va.) were coated for 24 h with 1 μg per ml of PBS of either affinity-purified goat anti-rat IgG antibodies (Zymed Laboratories, San Francisco, Calif.), affinity-purified rabbit anti-rat IgM antibodies (Zymed), monoclonal mouse anti-rat IgE antibodies (Serotec, Oxford, England), or 2 μg per ml of PBS of affinity-purified goat anti-rat IgA antibodies (Saxon Biochemicals). Bile and serum samples, which were diluted 1/500 for IgA and serum samples diluted 1/2,500 for IgG, 1/500 for IgM, and 1/10 for IgE antibodies, were further diluted in fivefold steps and incubated for 4 h at room temperature. For determination of IgG concentrations, alkaline phosphatase (APH)-conjugated goat anti-rat IgG antibodies (1/300; Zymed) were applied overnight. Thereafter, 100 μl of 1 mg of APH substrate (104 phosphatase substrate; Sigma, St. Louis, Mo.) per ml in 1 M diethanolamine buffer (pH 9.8) was added. The plates were read after 100 min on a spectrophotometer (Titertek Multiscan; Flow Laboratories, McLean, Va.) at 405 nm. For determination of the IgM, IgA, and IgE concentrations, a two-step ELISA technique was used. One hundred microliters of monoclonal mouse anti-rat IgM antibodies, mouse anti-rat IgA antibodies (1/3,000; Zymed), or goat anti-rat IgE antibodies (1/1,000; Nordic, Tillburg, Netherlands) was applied for 1 h at room temperature. The plates were then washed three times with PBS-Tween, and APH-conjugated goat anti-mouse Ig (1/4,000; Tago, Inc., Burlingame, Calif.) or APH-conjugated affinity-purified swine anti-goat Ig (1/1,000; Tago) was applied for 1 h at room temperature. The ELISA was developed with APH substrate, and the absorbance was read after 60 min in the spectrophotometer as described above. The concentrations of the immunoglobulins were derived from a standard curve obtained with IgG, IgM, IgA, or IgE myeloma proteins of known concentrations (Pharmingen, Stockholm, Sweden).

Determination of specific antibody levels. Microtiter plates were coated with 100 μl of bacterial lysate obtained from 10^7 boiled *E. coli* O6 K13 cells (26), 2 μg of O6 LPS per ml of PBS (prepared by phenol-water extraction [46]), or OA (5 μg/ml [Sigma]) overnight in room temperature. Bile samples were diluted 1/10 and serum samples were diluted 1/500 for IgG, 1/100 for IgM, or 1/10 for IgE in PBS-Tween and were diluted three more times in fivefold steps and added in 100-μl volumes to the wells. After an incubation time of 3 h, the plates were washed with PBS-Tween, and rabbit anti-rat IgG (1/10,000; Zymed), mouse anti-rat IgM, mouse anti-rat IgA (1/3,000; Serotec), or biotinylated mouse anti-rat IgE (1/1,000; Serotec) was applied for 1 h. Thereafter, 100 μl of APH-conjugated goat anti-rabbit IgG (1/10,000 [Sigma]), APH-conjugated goat anti-mouse Ig (1/4,000 [Tago]), or APH-conjugated streptavidin (1/1,000; Dako, Glostrup, Denmark) was applied for another hour. APH substrate was added as described above, and the absorbance was read in a spectrophotometer. The antibody activity was expressed in arbitrary ELISA units which were estimated from a standard curve obtained with a pool of hyperimmune sera.

The specificity of the assays was extensively tested to exclude cross-reactivities between different reagents.

Determination of OA. Microtiter plates were coated with 5 μg of an IgG fraction of goat anti-OA antibodies (Cappel, West Chester, Pa.) per ml of PBS. One hundred microliters of the supernatant from *E. coli* cultured in broth was applied, further diluted in twofold steps, and incubated for 3 h at room temperature. The plates were washed with PBS-Tween, and APH-conjugated anti-OA (1/1,000, Cappel) was applied overnight. The ELISA was developed with APH as described above. The absorbance values were compared with an OA standard.

Cytokine analysis. Cell suspensions from spleen cells at a final concentration of 2×10^6 cells per ml were seeded into 24-well plates (Nunc, Intermed, Denmark) and stimulated with 10 μg of concanavalin A (ConA, Sigma) per ml of cells for 3 days. The supernatants were collected and frozen at −20°C until analysis.

IL-2. Cells from the CTLL 2 cell line (American Type Culture Collection, Rockville, Md.) were cultured at a concentration of 2×10^4 cells per well with supernatants from ConA-stimulated spleen cells for 24 h. During the final 4 h, the cultures were pulsed with [^{3}H]thymidine (Amersham, Buckinghamshire, United Kingdom), harvested, and counted in a β-counter. A standard curve was established by using recombinant IL-2 (Genzyme, Cambridge, Mass.), and the contents of IL-2 in the supernatants were expressed in units per milliliter (22).

IFN-γ. IFN-γ was measured by ELISA. In brief, microtiter plates were coated with mouse monoclonal anti-rat IFN-γ (1/200 in PBS [Biosource International]). The plates were then blocked with 5% FCS for 30 min at 37°C, and supernatants from ConA-stimulated spleen cells were applied undiluted and further diluted in threefold steps and were incubated for 3 h. Rabbit anti-rat IFN-γ antibodies (1/10,000 in PBS-Tween and 5% FCS [Biosource]) were applied for 1 h, and thereafter APH-conjugated goat anti-rabbit IgG antibodies (1/2,000 in PBS-Tween and 5% FCS [Sigma]) were incubated for another hour. The ELISA was

TABLE 1. Various parameters in vitamin A-deficient and control rats 28 days after colonization[a]

Rat	Body wt at sacrifice	Vitamin A in serum (mmol/liter)	Vitamin A in liver (IU/g)	No. of bacteria/ g of feces (10^7)	Frequency of arthritis (%)
Vitamin A deficient (n = 8)	225 (15.4)	0.3 (0.2)[b]	<5[b]	36 (22)	75
Control (n = 8)	266 (11.9)	1.6 (0.5)[b]	1,500 (400)[b]	8 (4)	0
P	0.0008	0.003	0.001	0.004	0.0004

[a] Results are mean values (SDs).
[b] Mean from samples of four animals.

developed with APH substrate as described above and was read in a spectrophotometer. Recombinant IFN-γ (a gift from Peter van der Meide, TNO Institute of Applied Radiology and Immunology, Rijswijk, The Netherlands) was used as a standard, and the contents of IFN-γ in the supernatants were expressed in units per milliliter.

Statistics. The Mann-Whitney U test and the Student t test were used for statistical analysis.

RESULTS

Vitamin A levels. At the end of the experiment, the average serum retinol levels were 0.3 μmol/liter (standard deviation [SD] = 0.2; n = 4) in the vitamin A-deficient rats and 1.6 μmol/liter (SD = 0.5; n = 4) in the paired fed control animals. The average retinol levels in the liver were <5 IU/g (n = 4) for the deficient rats and 1,500 IU/g (SD = 400; n = 4) for the control rats (Table 1).

Body weight and clinical appearance. As a first clinical sign of vitamin A deficiency, about 4 weeks after the onset of the diet, the rats stopped gaining weight. At this time point, the average body weights of the deficient rats did not differ from those of the paired fed control rats. In contrast, 4 weeks after colonization with the *E. coli* strain, the vitamin A-deficient rats weighed significantly less than the control rats, despite equal intake of food (Table 1).

Additionally, 4 weeks after colonization, six of eight vitamin A-deficient rats had developed severe arthritis, which was defined as swelling and erythema of at least one joint, while none of the control rats had developed arthritis (Table 1).

Colonization of the gut with *E. coli* O6 K13 pOmp 21. Before antibiotic treatment, the normal flora of all rats was checked and none of the rats had *E. coli* O6 K13 in the gut. After the antibiotic treatment but before colonization, no aerobic bacteria were cultured from the rectal feces samples of any of the rats.

One day after colonization, the control rats had 50 times more bacteria in the cecum than the vitamin A-deficient rats. On day 3 after colonization, the number of bacteria per feces in the ceca of the deficient rats was five times higher than that in the control rats. This relation remained so until the end of the experiment (Table 1). Additionally, the vitamin A-deficient rats were colonized in all parts of the small intestine, while the control rats were colonized only in the ileum (Fig. 1).

Translocation of the bacteria. One day after colonization, pOmp 21 bacteria were found in the MLN of both groups but also in the blood and the livers from the deficient rats. Bacteria were also detected at days 3 and 7 in the Peyer's patches of deficient rats, but not in Peyer's patches from control rats. At sacrifice on day 28, bacteria were detected in six of eight MLN cultures from the vitamin A-deficient rats, while no bacteria were found in the MLN cultures of the control rats. The bacteria were identified as the *E. coli* pOmp 21 strain by the ability to produce OA. Four of the bacterial isolates from the kidneys could be identified as ampicillin-resistant *E. coli* O6 K13 strains; however, only two of them produced OA. Bacteria were found in two of the six arthritic joints from the deficient rats and were typed as ampicillin-resistant *E. coli* O6 K13 bacteria, but they did not produce OA.

Immunohistochemistry of the gut. The relative area expressing IgA-producing plasma cells in the lamina propria of the small intestine was about 5 times lower in the vitamin A-deficient rats than in the control rats (Fig. 2a). In contrast, the relative areas expressing $CD8^+$ cells and TGF-β-producing cells were approximately 10 times (Fig. 2b) and 3 times (Fig. 2c) higher in the laminae propriae of the deficient rats than in the laminae propriae of the control rats.

Concentrations of immunoglobulins in serum and bile. The total IgA concentrations in serum and bile did not differ between the two groups of rats, while the concentrations of IgG and IgM in serum were significantly higher in the vitamin A-deficient rats (Table 2). The concentrations of IgE in serum were higher in the vitamin A-deficient rats, although they did not reach a level of statistical significance.

Antibody levels in serum and bile. The vitamin A-deficient rats developed significantly higher levels of serum IgG antibody to the bacterial lysate and LPS antigens than the control rats after colonization (Fig. 3). This difference was increased after immunization with the bacteria. The IgM antibody levels against the bacterial lysate and LPS were two to eight times higher in the vitamin A-deficient rats than in the control rats. IgG and IgM antibody levels against OA did not differ between the groups before or after immunization; however, the levels were higher in both groups at the end of the experiment. Biliary IgA antibodies against the bacterial lysate and LPS did not differ between the groups, while the biliary IgA anti-OA antibody levels were significantly higher in the vitamin A-deficient group than in the control group (Fig. 4).

Interestingly, the vitamin A-deficient rats also developed significantly higher levels of IgE antibodies against the bacterial lysate and LPS, while no IgE antibodies against OA were

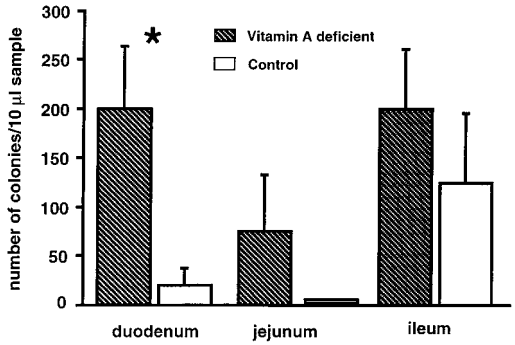

FIG. 1. Bacterial growth in different parts of the small intestine. The samples were taken with 10-μl sterile loops and cultured on tryptic soy agar plates containing ampicillin. The dark bars, means for five vitamin A-deficient rats; light bars, means for five control rats; error bars, standard errors of the means.

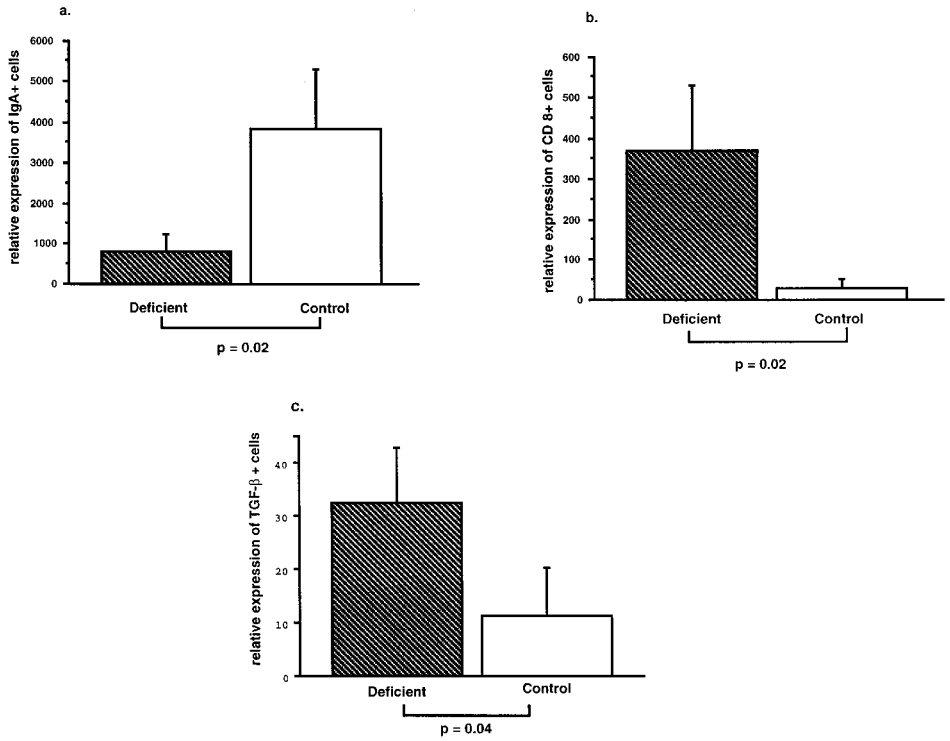

FIG. 2. (a) IgA-producing plasma cells in the laminae propriae of the small intestines of vitamin A-deficient and control rats. The relative expression levels reflect the ratio of the area of stained cells to the total area being counted. Dark bars, vitamin A-deficient rats; light bars, control rats. (b) $CD8^+$ cells in the laminae propriae of the small intestines of vitamin A-deficient and control rats. (c) TGF-β-producing cells in the laminae propriae of the small intestines of vitamin A-deficient and control rats.

detected (Fig. 5). Similarly to IgG, the IgE response to the bacterial lysate but not to LPS was increased after immunization with the bacteria.

Cytokines. IFN-γ levels in the supernatants of cultured spleen cells were generally rather low, but there was a significantly higher production in the cultures from the vitamin A-deficient rats (16.8 IU/ml [SD = 15.2; n = 8]) than in the cultures from the control animals (3 IU/ml [SD = 8.5; n = 8; P = 0.04]).

In addition, the IL-2 levels were significantly higher in the supernatants of ConA-stimulated spleen cells from deficient rats (0.44 IU/ml [SD = 0.25]) than in those from the control rats (0.05 IU/ml [SD = 0.11; P = 0.001]).

DISCUSSION

The present study describes three findings of major interest in response to bacterial colonization in vitamin A-deficient rats. Firstly, after an initially lower number of bacteria in the gut 1 day after colonization, the deficient rats had approximately five times more bacteria in their intestines than the control rats at the end of the experiment. There was also an increased translocation in the deficient animals. Secondly, the antibody responses against the bacterial antigens were higher in the deficient than in the control rats, and, thirdly, the vitamin A-deficient animals developed arthritis.

The association between vitamin A deficiency and infections is considered bidirectional in the way in which vitamin A deficiency decreases the resistance against infectious diseases and in which infections lead to poor nutritional status and changes in metabolism (8, 18, 19). Thus, the vitamin A-deficient rats in our study weighed significantly less than the control rats, despite equal food intake in both groups. Nutritional variables have been claimed to be important modulators of the gut barrier function and bacterial translocation (15). Bacterial translocation is defined as the presence of indigenous bacteria in MLN and other extraintestinal organs. Other significant factors influencing translocation are disruption of mucosal surfaces, bacterial overgrowth, and suppression of the immune system (4, 5). Vitamin A deficiency leads to impaired mucosal immune responses, as we have previously reported (47), a diminished number of mucus-producing goblet cells, and an increased vulnerability of the intestinal epithelium with an augmented risk of bacterial and viral penetration (2, 3, 35, 49).

TABLE 2. Immunoglobulin concentrations in serum and bile[a]

Rat	Serum IgG concn (mg/ml)	Serum IgM concn (μg/ml)	Serum IgE concn (μg/ml)	Serum IgA concn (μg/ml)	Biliary IgA concn (μg/ml)
Vitamin A deficient (n = 8)	3.3 (0.8)	51 (8.6)	21 (30)	16 (6.7)	225 (145.6)
Control (n = 8)	1.9 (1)	42 (4.5)	9 (12)	15 (4.5)	213 (64.8)
P	0.01	0.05	NS	NS	NS

[a] Results are means (SDs). NS, not significant.

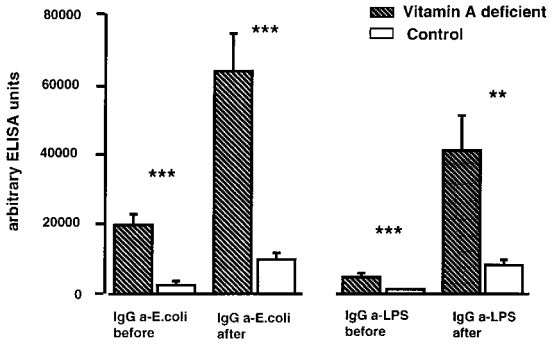

FIG. 3. Serum IgG antibody levels against the bacterial lysate of *E. coli* O6 K13 and LPS in vitamin A-deficient and control rats. Dark bars, means of 8 individual values for vitamin A-deficient rats; light bars, means for eight individual control rats. Error bars represent standard errors. The antibody levels were determined by ELISA and are expressed as arbitrary ELISA units. **, $P < 0.01$; ***, $P < 0.001$; before and after, before and after immunization with wild-type *E. coli* O6 K13, respectively.

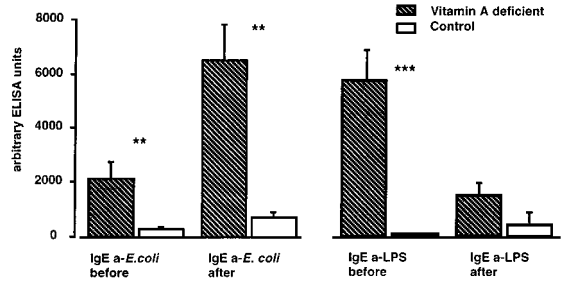

FIG. 5. Serum IgE antibody levels against the bacterial lysate of *E. coli* O6 K13 and LPS in vitamin A-deficient and control rats. Dark bars, means of eight individual levels from vitamin A-deficient rats; light bars, means for eight control rats; error bars, standard errors of the means. The antibody levels are expressed as arbitrary ELISA units. **, $P < 0.01$; ***, $P < 0.001$; before and after, before and after immunization with wild-type *E. coli* O6 K13, respectively.

Since the vitamin A-deficient rats had bacterial overgrowth in the small intestine, factors favoring high translocation were present in these animals.

The bacterial overgrowth and the increased translocation could have been influenced by changes in the adherence of the bacteria to the intestinal epithelia of the deficient rats. However, we were not able to demonstrate any difference in bacterial adherence to the enterocytes between the groups (data not shown). Therefore, this is probably not an explanation for the increase in translocation. Furthermore, nonadhering latex particles penetrate the gut epithelium readily, as reviewed by Berg (5), supporting the idea that adhesion might not be of the utmost importance for translocation.

The essential role of the immune system, in particular of T cells, in the control of bacterial translocation has been recently demonstrated (21). Those authors showed that depletion of $CD4^+$ and/or $CD8^+$ cells increased the translocation of bacteria from the intestinal flora, which is reflected by the presence of bacteria in the MLN. In the present study, we found an increased translocation of intestinal bacteria despite a higher number of $CD8^+$ T cells in the vitamin A-deficient rats. This suggests that the functional capacity of these cells was changed. In a previous study and in the current study, we have reported findings consistent with an aberrant T-cell function. Hence, T cells from vitamin A-deficient rats have an increased production of IL-2 and IFN-γ after both mitogen and antigen stimulation (48). It is known that IFN-γ has direct effects on the intestinal epithelium resulting in disintegration of tight junctions and decreased epithelial cell proliferation (1, 31). An increase in the production of IFN-γ by intestinal T cells might thus add to the destruction of the mucosal epithelium, promoting bacterial translocation. Furthermore, there was a higher number of TGF-β-producing cells in the laminae propriae of the vitamin A-deficient rats. Since TGF-β is also considered to act as a proinflammatory cytokine (43), the increase in the level of TGF-β could add to the inflammation in the intestine which might further favor an increase in bacterial translocation. At the same time, TGF-β may be immunosuppressive for T cells and may contribute to a defective local host defense (38).

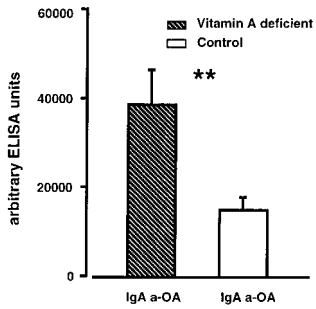

FIG. 4. Biliary IgA antibody levels against OA in vitamin A-deficient and control rats. Dark bars, levels for vitamin A-deficient rats (means of eight individual levels); light bars, means for eight individual levels from the control rats. The antibody levels are expressed in arbitrary ELISA units. Error bars, standard errors of the means; **, $P < 0.01$.

Previously, we have demonstrated that rats with ordinary gut flora and vitamin A deficiency have insufficient T_H2 activity, with inadequate subsequent antibody responses in secretory IgA, IgG, and IgE antibodies (48). These findings were confirmed insofar as the number of IgA-producing cells in the laminae propriae of vitamin A-deficient rats was significantly lower than that in control rats. However, the increase in translocation and the tissue invasion of the colonizing *E. coli* induced a systemic as well as secretory antibody response that was greater than or equal to the response of the control rats. We have previously shown that the antibody response in vitamin A-deficient rats is decreased only against T-cell-dependent antigens but not against T-cell-independent antigens (48). Moreover, it was recently reported that the antibody response to pneumococcal polysaccharide was augmented in vitamin A-deficient rats when it was coimmunized with LPS, a T-cell-independent antigen and polyclonal B-cell activator (36). The enhanced antibody response in the colonized vitamin A-deficient rats could therefore have been due to the bacterial exposure in the MLN and an adjuvant effect of the bacterial LPS. The increase in the concentration of immunoglobulins in the sera of the vitamin A-deficient animals supports the idea that the bacteria induced a polyclonal activation. In addition to the increased IgG, IgM, and IgA antibacterial antibody responses, the vitamin A-deficient animals developed high levels of IgE antibody against the bacterial antigens. This finding is of special interest, since only few studies have shown that bacteria can provoke IgE antibody responses (13, 14, 29). The high level of IgE antibodies would suggest an increased risk of inflammatory reactions in the intestine, with concomitant diarrhea or intestinal anaphylaxis. In agreement with this, we have seen in a subsequent study that colonized vitamin A-deficient rats have a higher frequency of diarrhea (unpublished results). In addi-

tion, intestinal anaphylaxis after intraluminal challenge with antigen has been recently demonstrated for mice with high levels of IgE antibodies (40).

Even if the antibody response was higher in the deficient rats, there were still higher numbers of bacteria in the MLN and kidneys and a subsequently higher frequency of arthritis. One reason for this high number of bacteria could be that the intracellular killing of bacteria by phagocytes was impaired in the vitamin A-deficient animals (unpublished data). Phagocytosis of bacteria by submucosal macrophages has been proposed to be a pivotal mechanism in controlling translocation (44).

Seventy-five percent of the vitamin A-deficient rats developed arthritis, while none of the control rats did. The etiology of the arthritis cannot be clearly defined, even though the most probable explanation is that the high number of translocated bacteria led to an invasion of the joints. The reason why bacteria could not be isolated from the joints in more than two cases might have been that the samples were taken at a late stage of the arthritic process, and thus the bacteria had already been cleared. The histology further indicated a chronic stage of the arthritis, since fibrotic reorganization and pannus formation rather than acute inflammatory reactions were seen in the sections (data not shown). A previous study of bacterial arthritis, supporting our suggestion, showed that *E. coli* cells that were administered intraarticularly were cleared from the joints within 72 h. Thereafter, the acute inflammation proceeded to a chronic stage despite the fact that the joints were sterile. The authors suggested that bacterial components, such as endotoxin, persisted in the joints and caused the chronic synovitis (7, 24). Alternatively, only certain bacterial antigens, such as LPS, could have entered the joints and elicited a reactive arthritis by attracting polymorphonuclear cells, macrophages, and T cells (27). Finally, we cannot rule out the idea that the arthritis was caused by anergic autoreactive T cells being activated by the high number of bacteria in the MLN. These T cells could have migrated to the joints, where they might have reacted with autoantigens and induced arthritis (28).

In conclusion, this is the first report showing that vitamin A deficiency leads to an increase in translocation of bacteria and an increase in the risk of infections such as septic arthritis. Furthermore, the current results suggest that vitamin A deficiency could be an etiological factor in chronic diarrhea, since a substantial number of cases of chronic diarrhea is associated with bacterial overgrowth in the small intestine and severe malnutrition. Clinical studies will be needed to investigate this possible connection.

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