Nutrition and Immunology

Decreased Resistance and Immune Response to Escherichia coli Infection in Chicks with Low or High Intakes of Vitamin A

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ABSTRACT  The effects of vitamin A excess or insufficiency on resistance to Escherichia coli infection and subsequent anti-E. coli immune responses were examined in chicks. Chicks receiving depleted (0 µg/kg), sufficient (0.85 mg/kg) or excess (1000 mg/kg) levels of vitamin A in their feed were inoculated by a subcutaneous injection of pathogenic E. coli (1 x 10⁸ and 2 x 10⁹ cfu per chick). Susceptibility to E. coli was determined by mortality, morbidity and immune responses (antibody production and T lymphocyte proliferation). Excess or insufficient vitamin A led to increased susceptibility of chicks to E. coli infection; this was accompanied by depressed immune responses. Chicks receiving excess vitamin A were more sensitive to E. coli than vitamin A-depleted chicks. This was reflected in higher mortality and morbidity rates and in severely depressed immune responses. In contrast to chicks receiving excess vitamin A, T lymphocyte responses (though not antibody responses) of vitamin A-depleted chicks achieved levels similar to those of vitamin A-sufficient birds with a lag period of 6 to 10 d. Therefore, reduction in resistance to E. coli infection, resulting from vitamin A excess or deficiency, probably was compounded by a delayed immune response. J. Nutr. 121: 395-400, 1991.

INDEXING KEY WORDS:
- chicks • vitamin A
- E. coli • infection • immune response

Vitamin A deficiency and excess is associated with depressed immunity [1-3]. Vitamin A deficiency is accompanied by low levels of serum immunoglobulins [4-6], impaired immunoglobulin (Ig)G [7, 8] and IgA responses [6], reduction in delayed-type hypersensitivity reactions [9-11], depressed responses to mitogens [12, 13] and reduced natural killer cell activity [14]. We previously showed that vitamin A insufficiency depressed in vitro T lymphocyte responses and in vivo antibody production to defined protein antigens [1]. Excess vitamin A decreased immune responsiveness in chicks to defined protein antigens [15], and high intakes of vitamin A were reported to decrease cellular immune functions in mice [16].

Whether or not a decreased immune response, resulting from deficient vitamin A, increases mortality and morbidity as a result of infections has not been established. Studies have shown that children deficient in vitamin A are more susceptible to disease than those receiving an adequate amount of the vitamin [17-19]. The relationship between vitamin A and infection in chickens has been investigated [20-22]. These studies described reduced resistance to infection associated with marginal vitamin A intake, but they did not discuss possible related immune functions or the effects of excess vitamin A.

The purpose of our study was to examine the effects of vitamin A excess or insufficiency on the capacity of chicks to resist a pathogenic Escherichia coli challenge and to evaluate the respective immune response.

MATERIALS AND METHODS

Animals. New Hampshire × Leghorn chicks were obtained from a local hatchery and maintained from the day of hatching in temperature-controlled brooders with free access to water and the experimental diet [15], which was supplemented with 0, 0.85 or 1000 mg/kg of retinol equivalent as retinyl acetate. Animals were weighed at weekly intervals.

Determination of retinol. Homogenized liver was saponified with ethanolic KOH and extracted into
petroleum ether as described by Vaisman et al. [23]. Retinol was determined by reverse-phase high performance liquid chromatography on a C18 column [Varian Instruments, Palo Alto, CA] using retinyl acetate as the internal standard [24].

**E. coli preparation and inoculations.** *E. coli* serotype 0:78K:80 was used in this study. The bacteria were grown in nutrient broth [Difco, Detroit, MI] for 16 h at 37°C and harvested at 5000 × g for 10 min at 4°C. The pellet was resuspended in sterile saline, washed three times and resuspended in 10 mL of saline. The number of bacteria were determined by comparing optical density of a saline suspension at 540 nm (Spectronic 70, Bausch & Lomb, Rochester, NY) with a previously calibrated standard. A suspension of 1 × 10^10 bacteria per milliliter was diluted (1:1) in glycerol and stored at −70°C until used. Thawed bacteria were seeded on McConkey agar [Difco, Detroit, MI] to establish the number of viable colony forming units at the time of inoculation. Bacterial death following freezing and thawing was less than 5%. Chicks were inoculated by a subcutaneous injection containing different levels of *E. coli* in 0.5 mL of saline.

**T lymphocyte proliferation assay.** The in vitro assay for antigen-specific proliferation of T lymphocytes in the chick has been described [15, 25]. In brief, peripheral blood leukocytes of chicks, separated by slow centrifugation (35 × g, 15 min), were suspended in Dulbecco’s modified Eagle’s medium supplemented with antibiotics, 1 mmol glutamine, 1% nonessential amino acids, 1% Na-pyruvate stock solutions [all from Biological Industries, Beit Haemek, Israel], 1 × 10^{-6} mol 2-mercaptoethanol [Sigma, St. Louis, MO] and 1% normal chicken serum. Leukocytes were cultured in 96-well, flat-bottom cluster plates [Costar, Cambridge, MA] in 200 mL of medium (1 × 10^4 cells per well) in the presence or absence of heat-killed *E. coli* [several doses ranging between 1 × 10^4 and 1 × 10^6 bacteria per well]. The cultures were placed in a humidified incubator containing 7.5% CO2 in air at 39°C. Following 92 h of culture, 37 kBq [1 μCi] of [3H]thymidine [specific activity 370 GBq/mmol [10 Ci/mmol], Nuclear Research Center, Dimona, Israel] was added to each well for 4 h. The cultures then were harvested onto filters by a multiharvester [Dynatech, Wesbart, England] and counted in a liquid scintillation counter with channel ratio quench correction calculating results as disintegrations per minute [Packard, La Grange, IL]. Counting efficiency was 55–60%. The results of cells from individual animals are the average of quadruplicate cultures and are expressed in disintegrations per minute. The average response of cells in the presence of medium alone (background responses) was 1100 dpm [range, 400–2100 dpm], the average response to *E. coli* of cells from nonimmunized birds was 900 dpm [range, 430–1500 dpm].

**Assay of antibody production.** Antibodies specific for *E. coli* were detected in sera of chicks by an enzyme-linked immunoassorbent assay (ELISA) [25]. In brief, dilutions of chick sera were added to microtiter plates coated with sonicated *E. coli* (10 μg protein per well). Dilutions of a negative serum were routinely added to all microtiter plates. After three washes to remove excess unbound antibody, the bound chick anti-*E. coli* antibodies were determined by using a peroxidase-rabbit anti-chicken IgG [heavy and light chain] [Bio Makor, Rehovot, Israel]. Nonspecific binding was blocked by chick serum that previously was shown to have no specific antibody activity. Peroxidase substrate ABTS, 1 component [KPL Inc., Gaithersburg, MD] was used for color development. The assay was read in a Bio-Tek ELISA reader [Bio-Tek Instruments, Winooski, VT] at 405 nm after 7 min of enzyme reaction at a constant temperature (21°C). The results of individual animals are the average of triplicate measurements and are expressed as a positive/negative ratio at a serum dilution of 1:400. The ratio was calculated by dividing the ELISA absorbance of the tested antiserum by that of the negative serum, which previously was shown to have no anti-*E. coli* binding [25]. The absorption values of the negative serum alwas were less than 0.2, and the same negative serum was used in all experiments.

**Statistical analyses.** Significance of differences (P < 0.01) was determined by analysis of variance using Duncan’s multiple range test for analysis of difference between groups [26].

**RESULTS**

To establish the effect of vitamin A nutrition on resistance of chicks to *E. coli* infection, preliminary experiments were performed to establish the pathogenicity and lethality of the inoculated bacterial strain. Vitamin A–sufficient chicks were inoculated by a subcutaneous injection with increasing levels of viable *E. coli* colony forming units. Ensuing mortality was recorded for 8 d and plotted against the respective inoculation (Fig. 1). In this experiment, mortality reached a plateau when levels higher than 4 × 10^9 cfu were injected; similar results were obtained in four additional experiments. We elected to examine the pathogenic effects of *E. coli* by using two concentrations of colony forming units that caused 20% and 50% morality rates (1 × 10^5 cfu per chick and 2 × 10^6 cfu per chick, respectively) in vitamin A–sufficient chicks.

Hepatic vitamin A status of experimental chicks was determined by 21 d of age and was 48 ± 5, 7 ± 3 and 750 ± 25 μg/g for vitamin A–sufficient, -depleted and –excess groups, respectively. The following experiments were initiated on d 21 and terminated within 10 to 20 d [31 to 41 d of age]. No physiologic manifestations of vitamin A excess or
depletion were observed within this time period [weight loss, eye lesions or bone fragility].

Twenty-one-day-old chicks receiving depleted [0 \( \mu \text{g/kg} \)], sufficient [0.85 mg/kg] or excess [1000 mg/kg] levels of vitamin A in their food were inoculated by a subcutaneous injection of pathogenic \( E. \text{coli} \) \( (1 \times 10^9 \) and \( 2 \times 10^9 \) cfu per chick). Mortality was recorded for 7 d, then the surviving chicks were inspected for \( E. \text{coli} \) infections of the liver and pericard (perihepatitis and pericarditis). Chicks with lesions were considered morbid. Each group contained 25 chicks, and the experiment was repeated seven times. The average means of these experiments (Fig. 2) shows that vitamin A–depleted and –excess birds were significantly \( (P < 0.01) \) less resistant than vitamin-A–sufficient birds to both doses of infective \( E. \text{coli} \). This was shown by increased mortality of the two experimental groups (Fig. 2, upper panel), by morbidity of the survivors (Fig. 2, lower panel) and by temporal mortality (Fig. 3, one experiment representative of seven). Birds fed excess vitamin A were most sensitive, particularly after receiving large lethal doses of \( E. \text{coli} \) (Fig. 2, \( 2 \times 10^9 \) cfu), all succumbed to disease by 96 h postinoculation (Fig. 3). Vitamin A–depleted birds were less sensitive; fewer birds died, but all survivors receiving \( 2 \times 10^9 \) cfu and 85% of those receiving \( 1 \times 10^9 \) cfu were morbid (compared with 50% and 30% in the vitamin A–sufficient group, respectively). The rate of death in vitamin A–depleted chicks was similar to that of vitamin-sufficient birds, but it occurred in larger numbers (Figs. 2 and 3).

We next evaluated whether or not reduced resistance to infection by pathogenic \( E. \text{coli} \) was accompanied by a parallel decrease in \( E. \text{coli} \)–specific immune responses. Chicks were inoculated with \( 2 \times 10^9 \) cfu pathogenic \( E. \text{coli} \). They were bled every 2 to 3 days thereafter to determine the presence of anti-\( E. \text{coli} \) antibodies in their sera (Fig. 4) and to determine the proliferative response of peripheral blood leukocytes to heat-killed \( E. \text{coli} \) (Fig. 5).

The antibody titer of vitamin A–depleted chicks increased with time, but it was significantly lower \( (P < 0.01) \) than the titer of vitamin A–sufficient chicks starting from d 8 postinoculation and throughout the experiment. The titer peaked sooner (d 8 compared with d 11) and diminished more rapidly than the respective titers of the vitamin A–sufficient group. Similar results were obtained in three consecutive experiments. In additional experiments, when fewer bacteria were injected \( (1 \times 10^9) \), antibody titers of depleted chicks were lower than those of vitamin A–sufficient chicks, but absolute titers were somewhat higher (not shown). Antibody titers of chicks receiving excess vitamin A and inoculated

**FIGURE 1** Mortality rates in vitamin A–sufficient chicks following inoculation with pathogenic \( E. \text{coli} \). Groups of 21-d-old chicks (25 chicks per group) were inoculated with different doses of pathogenic \( E. \text{coli} \) colony forming units. Mortality was scored for 8 d. Mortality, expressed as a percentage of group size, is the group mean; bars are SEM.

**FIGURE 2** Effects of vitamin A on resistance of chicks to pathogenic \( E. \text{coli} \) infection. Chicks given insufficient [–], sufficient [C] or excess [+]) vitamin A (21 d old, 25 chicks per group, seven experiments) were inoculated with \( 1 \times 10^9 \) or \( 2 \times 10^9 \) cfu \( E. \text{coli} \). Mortality was scored for 7 d. Mortality, expressed as a percentage of group size, is the average of means of seven experiments; bars are SEM (upper panel). Surviving birds were autopsied and scanned for pericarditis and perihepatitis. Chicks with these conditions were listed as morbid (lower panel). Morbidity, expressed as a percentage of surviving chicks within each group, is the average of means of seven experiments; bars are SEM.
with $2 \times 10^9$ cfu *E. coli* were not assayed for antibody production because chicks died within 3 to 5 d. Antibody responses of chicks with excess vitamin A surviving $1 \times 10^9$ cfu inoculation (less than 15%) were dramatically depressed; positive/negative ratios were 2 ± 1 and 4 ± 1, compared with positive/negative ratios of 8 ± 2 and 11 ± 3 of vitamin A-sufficient birds 6 and 10 d after inoculation, respectively ($P < 0.01$ for both groups).

The T lymphocyte proliferative response of vitamin A–depleted chicks receiving $2 \times 10^9$ cfu was severely depressed (Fig. 5) until 6 d after inoculation ($P < 0.01$ for d 3, 4 and 6). By d 9 and d 13 responses were restored to levels similar to those of vitamin A–sufficient chicks; from d 13 on the responses of both groups gradually decreased at the same rate (not shown). Similar results were observed in six additional experiments. Similar responses were obtained following the inoculation of $1 \times 10^5$ cfu, although the degree of T lymphocyte depression was less severe (not shown). T lymphocyte responses of chicks given excess vitamin A and receiving $2 \times 10^9$ cfu were not evaluated for the reasons stated here. The responses of survivors receiving $1 \times 10^9$ cfu were minimal until d 9 postinoculation ($1500 \pm 350$ dpm compared with $66,000 \pm 3500$ dpm for vitamin A–sufficient birds; $P < 0.005$) and increased slightly thereafter ($6000 \pm 1200$ dpm compared with $85,000 \pm 5500$ dpm for vitamin A–sufficient birds by d 15; $P < 0.005$).

**DISCUSSION**

In this study, excess or insufficient vitamin A led to increased susceptibility of chicks to pathogenic *E. coli* infection; this was accompanied by depressed immune responses. These results confirm and extend our previous studies showing depressed T lymphocyte and antibody responses to nonpathogenic protein antigens following excess or depleted vitamin A in the diet [15].
Increased resistance to *E. coli* infection in chicks receiving a short-term supplementation of vitamin A to an adequate diet has been reported (20), however, that study did not address the effects of vitamin A excess or depletion on resistance to infection or on immunoresponsiveness. Marginal vitamin A status in chicks was found to increase the severity of New Castle virus infections (22), but an examination of relevant immune responses was not attempted.

Susceptibility to pathogenic *E. coli*, determined in our study by mortality and morbidity, was accompanied by depressed anti-*E. coli* specific immune responses, as reflected by reduced antibody production and T lymphocyte proliferation. The degree of susceptibility and immune-response impairment was proportional to the dose of pathogenic bacteria inoculated. Note that all immune responses were assessed in chicks that survived the pathogenic challenge. This might have skewed results because animals with the most highly depressed immune functions probably succumbed to *E. coli* infection.

Chicks receiving excess vitamin A were more sensitive to pathogenic *E. coli* than vitamin A-depleted birds. This was reflected in higher mortality and morbidity and severely depressed immune responses as expressed by anti-*E. coli* antibody production and T lymphocyte proliferation. The deleterious effects of excess vitamin A on the immune response confirm and extend our previous findings (15) in which we employed a soluble, nonpathogenic protein antigen bovine serum albumin. The mechanism by which excess vitamin A depresses the immune response is not known. We previously suggested that this might result from the down-regulation of nuclear receptors for vitamin A or, alternatively, might be the result of toxic elevation of retinyl esters in the blood (15).

In contrast to chicks receiving excess vitamin A, T lymphocyte responses (though not antibody responses) of vitamin A-depleted chicks achieved levels similar to those of vitamin A-sufficient birds with a lag period of 6 to 10 d. Therefore, reduction in resistance to *E. coli* infection probably was compounded by a delayed immune response (1). Previous reports have shown that vitamin A increased in vitro blastogenesis of thymocytes by up-regulating receptors for interleukin-2 (27, 28). Therefore, vitamin A depletion might reduce the rate of in vivo T lymphocyte division and differentiation following antigenic stimulation. A similar case could be presented for B lymphocyte maturation, although antibody levels of depleted chicks did not achieve those of vitamin A-sufficient birds. Vitamin A has been shown to have direct effects on the differentiation of B lymphocyte hybridomas (29), but it also has been shown to have negligible effects on IgM production, which is considered to be independent of helper T lymphocytes (8). Therefore, our data, although not tested directly, could imply that IgM production was intact and IgG production was defective because production of IgG requires the involvement of helper T lymphocytes (30).

Reduced resistance to pathogenic *E. coli* also may involve other leukocytes. Decreased resistance to *E. coli* infection has been shown to be accompanied by reduced clearance of the bacteria from peripheral blood (Leitner, G. and Heller, E. D., personal communication). In addition, vitamin A has been shown to directly affect monocyte functions (31–34). Thus, the amount of vitamin A in the diet might affect the activity of several immune system cells and may not be limited to the malfunction of one cell type. Because the physiologic functions of these cells are different, a general metabolic regulatory pathway could be expected rather than a constitutive function of vitamin A; this theory is supported by the rapid recovery of immune functions following replacement of vitamin A in depleted rats and chicks (1, 30).

**LITERATURE CITED**


