Immune Responses of Female BALB/c and C57BL/6 Neonatal Mice to Vaccination or Intestinal Infection Are Unaltered by Exposure to Breast Milk Lycopene

Becky Adkins* and Nikhat Contractor

Abstract

Lycopene, a carotenoid produced by some commonly consumed plants such as tomatoes, is not synthesized by animals. Thus, the levels of lycopene found in the breast milk of lactating females reflect the dietary lycopene supply. Lycopene has potent antioxidant activity but has also been implicated in modulating immune function. Therefore, lycopene in breast milk has the potential to affect the development and/or function of the immune system in the suckling pups. Here, we have investigated the impact of breast milk lycopene on systemic and mucosal immunity in mouse neonates. Diets containing 0.3 g/kg lycopene (Lyc) or control (Con) diets were fed to mouse dams beginning at late gestation and continuing throughout lactation. Seven-day-old female BALB/c pups were parenterally immunized with a model vaccine antigen dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) and then reimmunized as adults. The levels of DNP-KLH–specific IgG in the sera as well as keyhole limpet hemocyanin-specific IFNγ and IL-4 production by splenic CD4+ cells were similar in the Lyc and Con pups. In addition, female neonatal (d7) C57BL/6 Lyc and Con pups were infected orally with the enteropathogen Yersinia enterocolitica. Breast milk lycopene had no effect on the recruitment of neutrophils to intestinal lymphoid tissues or on bacterial tissue colonization of the intestines, spleens, and livers. Thus, suckling pups exposed to lycopene in breast milk appear to develop normal innate and adaptive responses both systemically and at intestinal mucosal surfaces. J. Nutr. doi: 10.3945/jn.110.136762.

Introduction

The neonatal period of life is a challenging period immunologically. The infant is exposed for the first time to a vast array of antigens requiring an appropriate immune response. For example, infants must establish tolerance to the commensal microbiota while they simultaneously develop protective immunity against pathogenic microorganisms and respond to a large number of vaccine antigens. The immunological challenges to infant immunity are thought to be at least partially met by protective factors in the breast milk (1). Even partial breast feeding markedly decreases the risk of neonatal death from sepsis (2). In addition, breast feeding can enhance vaccine responses (3–5), facilitate the acquisition of tolerance to food antigens (6), and result in long-term protection against the development of asthma and atopy (3,7).

Studies in experimental animals to address the role(s) of milk components in neonatal immunity have generally consisted of increasing the amounts of normal milk compounds in the diets of suckling animals. For example, supplemental whey protein concentrates, containing many of the substances found in breast milk, have been shown to influence maturation of the intestinal immune system (8) as well as enhance the development of tolerance to food antigens (9) and increase resistance against rotavirus-induced diarrheal disease (10) in suckling rats. A more recent approach uses transgenic mice to deliver compounds naturally via the breast milk. Yen et al. (11) and Chen et al. (12) produced mice in which transgenic lactoferrin expression is confined exclusively to mammary gland tissue and secreted at high levels in the breast milk. Transgenic pups were protected from gastrointestinal infection with Escherichia coli, Staphylococcus aureus, Candida albicans, and enterovirus type 71. These studies indicate that breast milk components, when administered in excess, have the capacity to modulate neonatal immune development and function.

Studies in which neonates are exposed to supra-physiological levels of breast milk components are important for determining the potential of these compounds to affect the development of the

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1 Supported by the National Institute of Allergy and Infectious Diseases (grants AI044923 and AI083461 to B.A.) and Pfizer (B.A).
2 Author disclosures: B. Adkins, no conflicts of interest. N. Contractor is an employee of Pfizer.
3 Supplemental Figures 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
4 To whom correspondence should be addressed. E-mail: radkins@med.miami.edu.


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pups. However, they do not address the role of the naturally occurring amounts of these substances. An ideal setting for such a test would be to compare pups exposed to physiologically meaningful levels of a compound compared with no exposure at all. In this way, any effect(s) of the normal amounts of the breast milk components would be revealed. Using such an approach, we analyzed the impact on the neonatal immune system of lycopene, a natural component of human milk. Lycopene, a carotenoid, is a natural pigment synthesized by plants (e.g. tomatoes) and microorganisms but not by animals. The levels of lycopene found in the breast milk reflect the dietary carotenoid supply (13). Lycopene is a potent antioxidant and is perhaps best known for its potential role in the prevention of cancer (14,15). In addition to its antioxidant properties, there is increasing evidence that lycopene may have potent effects on immune function. Lycopene has been shown to exhibit antiinflammatory activity by directly downmodulating proinflammatory cytokine production and by interfering with the maturation of dendritic cells, at least in part by the inhibition of NF-κB activation (16–18; N. Contractor, unpublished data). However, in some contexts, lycopene has been found to enhance T helper cell (Th)1 cytokine responses (19,20) and may either enhance (19) or suppress (20,21) Th2 function. Together, these observations led us to propose that lycopene in the diet of nursing dams may potentially affect innate or adaptive immunity in the pups.

**Materials and Methods**

**Preparation of lycopene-containing feed.** Mouse nonpurified diet was formulated at Test Diet Laboratories. Modified LabDiet 5010 autoclavable feed (246 g/kg protein, 48 g/kg fat, 41 g/kg fiber, 17 MJ/kg gross energy) with reduced fortified vitamin levels was used as the base feed. The vitamin levels were reduced, because the diet was not autoclaved but sterilized by irradiation. In addition, reduced vitamin levels permitted us to better interrogate the effect of lycopene in the absence of potentially confounding effects, particularly from vitamin E. The levels of specific vitamins were: vitamin A acetate, 4.50 μg/g; cholecalciferol (in purified form), 0.13 μg/g; α-tocopherol acetate, 0.018 mg/g; and menadione, 1.9 μg/g. The base feed was supplemented with LycoVit 10% DC (BASF) to a final concentration of 0.3 g/kg lycopene and irradiated. This level of lycopene supplementation was chosen based on a previous report indicating that pups nursed on dams fed this amount of lycopene had transient increases in splenic T and B cells (22). Control feed consisted of the same modified LabDiet 5010 base feed with no additional supplements. All feed was treated steriley and stored at −80°C in the dark. Carotenoid analyses (Craft Technologies) revealed an actual lycopene concentration of ~0.25 g/kg feed.

**Mice.** Our neonatal models for adaptive and innate immune responses in neonates have largely focused on BALB/c and C57BL/6 mice, respectively. Therefore, BALB/c mice were used to study adaptive immunity (Expt. 1); C57BL/6 mice were used to study innate immunity (Expt. 2). Mice were bred and housed under barrier conditions in the Division of Veterinary Resources at the University of Miami Miller School of Medicine. Timed matings were set up for 24 h and the day of separation of the females from the males was considered d 0 of gestation. On d 16–18 of gestation, pregnant mice were placed on control (Con) or lycopene-containing (Lyc) diets. Dams continued to receive these diets until the pups were weaned (3 wk of age). At weaning, the pups were placed on the Con diet. At the indicated times, mice were killed by CO2 asphyxiation followed by cervical dislocation. All animal procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Miami Miller School of Medicine.

**Preparation of samples for lycopene concentration determination.** Sera from BALB/c dams fed Lyc or Con diet were collected at the time of weaning of the pups. Seven–day-old BALB/c female pups suckling on dams fed the Lyc or Con diet were killed and stomachs and livers were removed and flash frozen in an isopropanol/dry ice slurry. Tissues and fluids were analyzed for lycopene content by Craft Technologies.

**Protein antigen immunization.** Female neonatal (d7) BALB/c mice born to dams fed Con or Lyd diets were immunized with 25 μg dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH; Calbiochem) in PBS and then reimmunized 4–5 wk later with 100 μg DNP-KLH, as previously described (23).

**Cytokine ELISA, serum ELISA, and enzyme-linked immunospot assays.** Detailed descriptions of these procedures can be found in previously published material (23–25).

**Bacterial infection.** *Yersinia enterocolitica* A127/90 yopP serotype 08/ biotype IB was kindly provided by Guy R. Cornelis, Universitat Basel, Basel, Switzerland. D7 female C57BL/6 neonates born to dams fed a Lyc or Con diet were i.g. infected with 2 × 10⁷ CFU of *Y. enterocolitica* A127/90 yopP, as previously described in detail (26).

**Bacterial enumeration from organs of infected mice.** Intestines, spleen, and livers from C57BL/6 pups born to dams fed a Lyc or Con diet were prepared and homogenized. Bacterial titers were measured by plating on *Yersinia* Selective Agar plates (DIFCO) as previously described in detail (26). Control experiments with age-matched, uninfected mice demonstrated that intestinal commensal bacteria were undetectable using this selective medium (27) (data not shown).

**Cell staining, antibodies, and flow cytometry analyses.** The staining of mesenteric lymph node (MLN) cells to detect Ly6G^+^CD11b<sup>hi</sup> neutrophil phenotype cells was performed as previously described (26).

**Statistical analyses.** All cytokine and antibody responses (Expt. 1) were tested for significance using unpaired Student’s t tests; Mann Whitney nonparametric analyses were used for Expt. 2. Differences were considered significant at *P* < 0.05.

**Results**

**Lycopene does not affect vaccine-specific adaptive immunity in mouse neonates (Expt. 1).** Sera sampled from 2 BALB/c dams fed the Lyc diet contained 0.782 and 0.639 μmol/L of lycopene (data not shown). Interestingly, these values are more than 10 times greater than the concentration of lycopene found in the breast milk of nursing mothers around the world (13). Lycopene was additionally detected in the stomachs (0.086 and 0.080 μmol/g) and livers (0.982 and 0.931 μmol/g) of d7 BALB/c female pups nursed on the dams fed the Lyc diet but not in the pups nursed on dams fed the Con feed. These results confirmed that the lycopene in the feed can be absorbed by the nursing dams and transferred to the suckling pups via the breast milk.

For Expt. 1, we tested whether lycopene in the dams’ diets affected memory CD4<sup>+</sup> Th1/Th2 responses in the pups (Supplemental Fig. 1A). As determined by ELISA, the Lyc and Con groups did not significantly differ in IFNγ or IL-4 production or in the ratio of IFNγ/IL-4 (data not shown). Consistent with the ELISA experiments, enzyme-linked immunospot assay analyses revealed that the frequencies of antigen-specific CD4<sup>+</sup> cells secreting IFNγ or IL-4 were not different in the Lyc and Con groups (data not shown). In addition, anti-DNP IgG1 and IgG2a responses did not differ between the 2 groups in the titration

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8 Abbreviations used: Con, pups born to and nursed on dams receiving control (non-lycopene-containing) nonpurified mouse diet; DNP-KLH, dinitrophenyl-keyhole limpet hemocyanin; Lyc, pups born to and nursed on dams receiving lycopene-containing nonpurified mouse diet; MLN, mesenteric lymph node; Th, T helper.
curves of the sera (Fig. 1A,B) or the titers of the sera from individual mice (Fig. 1C,D).

To ensure that changes in antibody isotypes could be elicited in our system, we supplemented the immunization cocktail with CpG, which has previously been reported to enhance neonatal specific IgG2a production (28,29). CpG treatment elicited IgG2a antibody responses 9- to 27-fold greater in magnitude compared with no CpG treatment in both the Con and Lyc pups (Supplemental Fig. 2). Together, these experiments indicate that lycopene in the breast milk does not enhance but also has no adverse effect on neonatal systemic adaptive immunity.

**Innate intestinal immunity and bacterial growth in neonates are unaffected by lycopene (Expt. 2).** Although lycopene had no effect on systemic adaptive immune responses, we considered the possibility that we might see effects of lycopene in pups under conditions of high oxidative stress. Therefore, we turned to an infectious setting in which pups are i.g. infected with the Gram-negative enteropathogen Y. enterocolitica (Supplemental Fig 1B). We have found that pups infected with the A127/90 yopP strain of Y. enterocolitica develop extensive neutrophilic infiltration of the MLN (B. Adkins, unpublished data). This infiltration is accompanied by damage to the intestinal tissue and highly elevated levels of the enzyme myeloperoxidase, a marker of neutrophil activity (30). These observations are consistent with a condition of marked oxidative stress in the intestines of neonates infected with Y. enterocolitica yopP.

Five days postinfection, bacterial titers in the intestines, liver, and spleens of the Lyc and Con pups were evaluated (Fig. 2). The intestines of the Lyc pups contained more \( P = 0.046 \) bacteria than the that of the control group, but the groups did not significantly differ in colonization of the liver or spleen. There were also no significant differences in the percentages of neutrophils, identified as Ly6G+CD11bhi, in the MLN of the Con and Lyc pups (data not shown). Similar results were obtained at 3 d postinfection (data not shown). Together, these results demonstrate that lycopene in the breast milk does not interfere with the normal course of colonization by the Gram-negative enteropathogen Y. enterocolitica in neonates or with the activation of the intestinal innate immune system.

**Discussion**

Neonatal immune responses differ markedly from adult immune responses. In particular, in vitro and in vivo activation of neonatal T cells results in skewed production of Th2-type cytokines compared with activated adult T cells [reviewed in (31)]. As the neonate ages, this skewed response gradually matures into a more adult-like, Th1-dominant response. The shift in Th cell responses may be mediated by many factors, including establishment of the commensal microflora, exposure to pathogens, and exposure to immune modulating factors via the mother’s milk. In fact, cytokines present in the breast milk have been shown to inhibit the development of allergy in neonates (32). In addition to the cytokines present in breast milk, the neonate may be exposed to potential modulators of the immune response, such as lycopene, which are derived from the mother’s diet. To assess the role of mother’s milk derived compounds in the development of neonatal primary and secondary immune responses, it was necessary to determine whether nursing dams could indeed transfer a dietary compound to the pups via the breast milk. This would obviate the need for dietary intervention via oral gavage or systemic injection of the pups. This is important because of the potential variables associated with manipulating neonates such as possible rejection by the dams of the handled pups, whether the trauma of the manipulation might influence the results, and whether single daily doses by gavage or injection would be reflective of the multiple doses received at regular feeding intervals. We were able to demonstrate that pups nursed on dams exposed to lycopene via the diet had detectable levels of lycopene in the stomachs and

**FIGURE 1** Female serum IgG1 (A,C) and IgG2a (B,D) concentrations (A,B) and titers (C,D) in female BALB/c pups immunized twice with DNP-KLH and nursed on dams receiving the Con or Lyc diet. Numbers on the x axes indicate serum dilutions. In A and B, data are mean ± SD, \( n = 6 \) (Lyc) or 7 (Con). Relative titers in each serum sample were calculated as the lowest dilution giving a value greater or equal to the mean of all samples at the highest dilution plus 3 SD. In C and D, each symbol indicates an individual mouse. Means are indicated by a solid line.

**FIGURE 2** Intestine (A), liver (B), and spleen (C) bacteria concentrations in female C57BL/6 pups i.g. infected with Y. enterocolitica ΔyopP and nursed on dams receiving the Con or Lyc diet. Each symbol indicates an individual mouse. Means are indicated by a solid line. The dashed line indicates the limit of detection.
livers, whereas pups nursed on dams fed the Con diet had no detectable levels of lycopene. These studies are the first to our knowledge to address the role of lycopene delivered in a biologically relevant context (via the breast milk) in both systemic and innate responses of neonates.

Lycopene has been shown to alter Th cell responses in adult mice in a number of experimental settings (19–21). To understand the role of lycopene in the systemic immune response of neonates, mouse pups were immunized with a model vaccine antigen, DNP-KLH, and adaptive memory responses were assessed after re-challenge. In this system, the antigen-specific recall response was previously demonstrated to be diminished in Th1 function and instead biased toward a Th2 response (23,25,33,34). We anticipated that dietary lycopene might enhance the vaccine-specific Th1-mediated response. However, dietary lycopene did not measurably affect the normal development of Th2-dominant DNP-KLH–specific adaptive immune responses in the pups. It is unclear how lycopene mediates the immune effects that have been observed both in vitro and in vivo in adult mice. Thus, it remains possible that the mechanisms by which lycopene mediates its effects in adults are not present in neonates.

To investigate the potential role of lycopene in the innate immune response of neonates, mouse pups were exposed to a mucosal pathogen, Y. enterocolitica. Dietary lycopene had no effect on bacterial load at either 3 or 5 d postinfection and had no effect on intestinal neutrophilia. This was surprising given the observation that dietary supplementation with lycopene in adult mice modulates respiratory burst activity in peripheral blood neutrophils (35). Although neutrophil burst was not measured in our experiments, an alteration in neutrophil activity should result in a difference in bacterial colonization. Because no differences in bacterial colonization were observed between the Lyc and Con groups, it is unlikely that neutrophil respiratory burst activity was affected in the Lyc pups. The lack of an effect in our system could be explained in several potential ways. First, we chose to use a dietary level of lycopene based on the previous demonstration of an effect at this level (22). However, it remains possible that the levels of lycopene attained in the pups were not high enough to influence the physiological events we measured. Second, the breast milk itself may possess modulating properties that minimize or negate any influence of the lycopene in the offspring.

In summary, we used novel mouse models that mimic the natural exposure of newborn humans to lycopene to study the effects of this compound on immunity in pups. Although these studies do not cover the entire range of potential immune responses affected by lycopene, they do, importantly, demonstrate that dietary intake of lycopene by nursing mothers has no adverse effect on the systemic adaptive immune response to a model vaccine antigen or on the innate immune response to infection with a mucosal pathogen.

**Acknowledgments**

We thank Revati Darp, Patricia Guevara, and Emily Schwartz for outstanding technical assistance. B.A. and N.C. designed research; B.A. and N.C. performed data analysis; B.A. and N.C. wrote the paper; and B.A. and N.C. are responsible for the final content. Both authors read and approved the final manuscript.

**Literature Cited**


