Immunologic memory is established in nursing rats immunized with tetanus toxoid, but is not affected by concurrent supplementation with vitamin A¹–³

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ABSTRACT Experiments were conducted to determine whether nursing rats immunized with tetanus toxoid (TT) are able to produce a specific antibody response and whether oral treatment with retinyl palmitate, concurrent with immunization, affects the magnitude of the anti-TT response. When rats aged 8–15 d and nursed by vitamin A–sufficient dams were immunized with TT, no primary anti-TT immunoglobulin (Ig) M or IgG response was detected. However, nursing rats formed immunologic memory to TT because, when they were reimmunized at 40 d of age, their secondary anti-TT IgG response exceeded the primary response of 40-d-old vitamin A–sufficient rats (P < 0.02). Provision of retinyl palmitate (equal to 37.5 or 150 μg retinol equivalents) by mouth with early primary immunization did not change the magnitude of the secondary anti-TT IgG response. However, the age of nursing rats at first immunization significantly affected the magnitude of their secondary anti-TT IgG response, because rats first immunized at 15 d of age and reimmunized at 40 d of age produced a secondary response that was nearly fivefold greater than that of rats immunized at 8 and 40 d of age. In conclusion, nursing rats immunized with TT formed immunologic memory, which was affected significantly by the timing of the primary immunization. However, the administration of retinyl palmitate concurrent with early primary immunization did not significantly affect the development of memory to TT. Am J Clin Nutr 1995;62:1007–12.

KEY WORDS Vitamin A, retinyl palmitate supplementation, tetanus toxoid, antibody response, immunologic memory, Lewis rat

INTRODUCTION Since the 1920s, a preponderance of experimental evidence has accumulated that vitamin A is essential for both humoral and cell-mediated immune processes, including T cell–dependent and –independent antibody responses to specific antigens, T cell proliferation in response to nonspecific stimuli, and natural killer cell activity (1, 2). Field-based epidemiologic studies have demonstrated a strong relation between vitamin A deficiency and increased childhood morbidity and mortality (3–5). Such studies have also shown that the incidence and severity of infection and the risk of mortality are significantly diminished by the provision of vitamin A to children with marginal vitamin A status (6–8).

Neonatal tetanus, resulting from infection with the antigenic component of the bacterium Clostridium tetani, remains a significant public health concern in developing countries, causing > 750 000 deaths annually (9). Given that many countries have developed an effective infrastructure for child immunization, it is attractive to consider including vitamin A supplementation during field contacts designed to immunize young children against tetanus and other diseases. Clinical trials and experimental studies have been initiated to determine whether the provision of vitamin A concurrent with child immunization may be effective in preventing, or lessening, the incidence and severity of infection in children at high risk for developing vitamin A deficiency. Recently, Semba et al (10) reported the results of a large, randomized, placebo-controlled clinical trial in which the primary antitetanus IgG response of 3–6-y-old Indonesian children was enhanced significantly when 60 000 μg retinol equivalents of vitamin A was given near the time of diphtheria-pertussis-tetanus (DPT) immunization. Similarly, in young vitamin A–deficient rats, the primary and secondary antitetanus toxoid (TT) responses were significantly increased when a large oral dose of retinyl palmitate was administered near the time of immunization (11, 12).

It has been proposed that immunization programs in developing countries be designed so that children, including infants as young as 6 wk of age, receive vitamin A at the time of DPT immunization (13). Such administration would be mainly prophylactic, because frank deficiency is very rare at this age. However, adequate studies are lacking on whether vitamin A administration, at an early age and concurrent with immunization, has any effect on the antibody response. It is difficult to predict what effect vitamin A might have, because several possibilities are plausible. One possibility is that vitamin A supplementation very early in life will have no observable effect on antibody responses, because no vitamin A deficiency yet exists. A second possibility is that antibody responses might be greater, based on the adjuvant activity of vitamin A in

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some circumstances (2), and the speculation that retinol or its metabolites is involved in T cell activation (14) and B cell growth (15). Yet another possibility is that vitamin A administration might activate immune responses, yet depress the antibody response. Such speculation is suggested by reports that a diet very high in retinyl acetate resulted in activation of graft rejection in neonatal mice that are normally immunologically tolerant to histoincompatible tissues (16, 17). The lymphocyte profile of young children and animals changes significantly in the postweaning period. In an earlier study with nursling rats, we found that the ratio of CD8+ to CD4+ T cells was elevated, consistent with low antibody responses to T cell–regulated antigens in young rats (18). It is therefore conceivable that vitamin A provided with early immunization might activate immune cells that favor suppression, rather than development, of the primary antibody response or the formation of immunologic memory.

With these numerous considerations in mind, we designed the present studies using young rats as a model to explore, first, whether preweaning rats immunized with TT at an age comparable with that of a human infant are able to produce detectable primary and secondary antibody responses and, second, whether concurrent treatment with vitamin A affects the development of immunologic memory as assessed by measuring the secondary response to TT after reimmunization of rats in young adulthood. During the course of these studies, we became aware that small differences in ages of nursling rats at the time of primary immunization might significantly affect the magnitude of their secondary anti-TT IgG response; thus, an additional study was designed to test this hypothesis.

MATERIALS AND METHODS

Animals, diets, and experimental design

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Pennsylvania. Female Lewis dams (virus antibody-free) with male pups were purchased from either Charles River Breeding Laboratories (Wilmington, MA) or Harlan Sprague Dawley (Indianapolis). All rats were fed a semisynthetic diet, described previously (19, 20), that contains an adequate amount of vitamin A [4 µg retinol equivalents (RE) as retinyl palmitate] per gram of diet. All rats had free access to food and drinking water and were housed in a rodent facility at 22 °C with a 12-h light-dark cycle. Soon after their arrival, pups were redistributed randomly among dams so that each litter contained between 8 and 10 pups.

The data represent the results of three separately conducted experiments: experiments 1 and 2 were of the same design (except that experiment 2 included a low dose of oral vitamin A, see below) and appropriate data from these two experiments have been pooled for presentation; experiment 3 differed in design from the first two experiments with regard to the ages of nursling pups at the time of primary immunization with TT. In experiments 1–2, pups were specified by the supplier to be 2–5 d old on arrival, but their exact ages were unknown to us. These rats received their first immunization with TT (see below) when they were ~12 d old, but they may actually have been from ~10 to 14 d old at this time. As is discussed in the Results section, it became apparent to us that even these small differences in the ages of nursling rats when immunized might have a significant effect on their secondary anti-TT response. Thus, experiment 3 was designed to confirm and extend experiments 1–2 by examining the effect of age at primary immunization on the magnitude of anti-TT IgG production. For experiment 3, we purchased nursling rats that were born no more than 1 d apart and whose exact ages were known to us; these nursling pups were primed with TT when they were either 8 or 15 d old.

Nursling rats designated as retinyl palmitate–supplemented were treated on the day of primary immunization with an oral dose of retinyl palmitate, equal to either 37.5 RE (experiment 2) or 150 RE (experiments 1–3). These doses were chosen to be equivalent to 6250 and 30,000 RE (25,000 and 100,000 IU vitamin A), respectively, in a child weighing ~5 kg. Retinyl palmitate (lot 121H0536; Sigma, St Louis) in 15 µL sunflower oil (SFO) was delivered with a plastic micropipette tip on the back of the tongue. Nursling rats designated as nonsupplemented were given a comparable amount of SFO.

Immunizations, blood collection, and antibody assays

Nursling rats were immunized intraperitoneally with 10 µg TT (lot 297 or 292; Connaught Laboratories, Ontario, Canada) in 200 µL sterile saline. For the primary or secondary immunization of 40-d-old rats, 100 µg TT in 1 mL sterile saline was used. For each age group, a small number of nonimmunized rats was injected with a comparable amount of sterile saline, and their plasma was analyzed to assess any background reactivity in the anti-TT IgM and IgG assays.

In all studies, ~50–200 µL blood was drawn from either the caudal vein or the inferior vena cava into heparinized syringes. Plasma obtained by centrifugation at 600 × g for 20 min at 4 °C was stored in aliquots at −20 °C until anti-TT IgM or IgG concentrations were determined. After primary immunization of nursling pups with TT, plasma was collected after 4, 7, 10, and 14 d for analysis of anti-TT IgM and IgG. After the primary or secondary immunization of 40-d-old rats, plasma was collected 10 d later because antibody responses had previously been shown to have reached their maxima at this time (12, 21).

The enzyme-linked immunosorption assay procedure used for the detection of anti-TT IgM and IgG was described in detail elsewhere (12). Briefly, Immunolon 4 polystyrene flat-bottom 96-well plates (Dynatec Laboratories, Inc, Chantilly, VA) were coated with 10 mg TT/L prepared in Tris–HCl buffer (TB buffer), pH 10.6 for IgM or pH 7.6 for IgG. Plates were washed and incubated with bovine serum albumin (BSA)-TB buffer to block any nonspecific binding sites. After the BSA was removed, appropriately diluted plasma samples were added to each well and the plates were incubated at 4 °C overnight. The wells of each plate were washed and incubated with an appropriate amount of either diluted alkaline phosphatase–conjugated goat antirat IgM or goat antirat IgG (Cappel Scientific, Westchester, PA). The plates were then washed and color was developed by adding p-nitrophenyl disodium phosphate substrate (Sigma Chemical Co) to each well. Absorbance was read at 405 nm (reference wavelength of 570 nm) on an automated microplate reader (model MR 600; Dynatec Laboratories). As an internal control, each plate contained a serially diluted standard plasma sample from a rat immunized twice with TT. Absorbance units were converted to either milligrams of anti-TT IgM/L plasma or milligrams of...
anti-TT IgG/L plasma (12) by using this standard plasma sample.

Tissue total vitamin A analyses

Total vitamin A concentrations in plasma and liver samples were quantified after saponification and analysis by HPLC, described previously (19).

Statistics

Data are expressed as the mean ± SEM. When the variance among treatment groups was unequal, the Mann-Whitney U test was used for statistical analysis; however, when the variance among treatment groups was equal, an unpaired t test or Bonferroni multiple-comparisons test after one-way analysis of variance (ANOVA) was used (INSTAT program; Graph Pad, San Diego). Statistical significance was accepted at \( P \leq 0.05 \).

RESULTS

Plasma anti-TT IgM or IgG responses in ~12-d-old nursing rats immunized with TT

When young rats, ~12 d old and nursed by vitamin A–sufficient dams, were immunized with TT and their plasma was collected 4, 7, 10 (n = 6/d), and 12 d (n = 1) after immunization, no specific anti-TT IgM or IgG response was detected (data not shown). In contrast, and as a positive control, the primary responses of older animals were readily detectable (see below).

Although vitamin A–sufficient rats immunized at an early age did not produce a measurable primary antibody response to TT, we next questioned whether there might still be a response in the form of the development of immunologic memory. If so, a secondary anti-TT IgG response should be measurable after rats primed at an early age are reimmunized as young adults. Because high doses of vitamin A have been shown to have a stimulatory effect on some immune responses (16, 17), we also examined the effect of a single oral dose of 37.5 or 150 RE retinyl palmitate, given at the time of the primary immunization on the secondary plasma anti-TT IgG response.

In experiments 1–2 (Figure 1), nursing pups were primed with TT when they were ~12 d old and some were treated with retinyl palmitate; all rats were reimmunized with TT as young adults (when ~40 d old). In parallel, other vitamin A–sufficient 40-d-old rats received a primary immunization with TT at this time. Rats primed as nurslings and reimmunized when ~40 d old (Figure 1A) produced an anti-TT IgG response that was significantly greater than the response of rats first immunized when they were 40 d old (Figure 1B). Therefore, we infer the antibody response of rats primed at ~12 d of age to be a secondary, rather than a primary, response. Figure 1A also shows that this secondary anti-TT IgG response was not affected by the prior treatment of nursling rats with either dose of retinyl palmitate. This lack of effect of supplemental retinyl palmitate on the secondary anti-TT response (Figure 1A) cannot be attributed to insufficient sample size because power analysis indicated that a minimum of 13 rats/group was required to achieve 80% power with a two-tailed \( t \) test. As is shown in Figure 1A, the three treatment groups each included 13–24 rats. Thus, the lack of statistical significance between the secondary anti-TT responses of supplemented and non-supplemented rats can be interpreted as a truly negative result.

Age at priming with TT affects the magnitude of the secondary anti-TT response

During the course of experiments 1–2 in which nursling rats were primed with TT at ~12 d of age, we observed a tendency for the secondary anti-TT response (after reimmunization when rats were ~40 d old) to be lower if rats had been smaller as nurslings (and therefore estimated to be nearer to 10 d old when first immunized) than if they had been larger (estimated to be nearer to 14 d old when first immunized). However, because no firm conclusion could be drawn regarding the possible effect of age, experiment 3 was conducted with nursling rats that were known to be exactly 8 or 15 d old at the time of their initial immunization and treatment with retinyl palmitate. Although all of the rats produced a measurable anti-TT response after they were reimmunized at 40 d of age (Figure 2A), the age of nursling rats when they were primed with TT had a significant effect on the magnitude of their secondary anti-TT IgG response. The mean plasma anti-TT IgG concentration of 40-d-old rats was about six- to eightfold greater (\( P < 0.02 \)) in rats given a priming immunization when they were 15 d old than when they were 8 d old (Figure 2A). As was observed in...
experiments 1–2, the secondary antibody response, measured 10 d after reimmunization, was 4–30-fold greater (P < 0.01) than the primary anti-TT IgG response of age-matched, nonsupplemented rats (Figure 2B). Also consistent with experiments 1–2, the treatment of either 8- or 15-d-old nursling rats with 150 RE retinyl palmitate did not affect the magnitude of their secondary anti-TT IgG response.

Vitamin A status after retinyl palmitate supplementation

To confirm that the vitamin A supplement resulted in differences in vitamin A status during the primary response to antigen, plasma and liver total vitamin A concentrations were measured in a small number of retinyl palmitate–supplemented and nonsupplemented rat pups 2 d after primary immunization. Table 1 shows that retinyl palmitate supplementation of rats in all age groups resulted in liver total vitamin A concentrations that were about two- to fourfold higher than those of nonsupplemented rats (P < 0.001 for rats supplemented with 150 RE as retinyl palmitate when 12 d old). In experiment 2, a lower oral dose, 37.5 RE retinyl palmitate, also produced an increase in liver total vitamin A (P < 0.002, Mann-Whitney U test). Plasma retinol concentrations were not significantly elevated 2 d after supplementation with either dose of retinyl palmitate. None of the rats exhibited any signs of vitamin A toxicity from these doses.

DISCUSSION

Vaccination with T cell-dependent, soluble protein antigens such as TT elicits the activation, proliferation, and differenti-

<table>
<thead>
<tr>
<th>Age when treated</th>
<th>Supplement given</th>
<th>Plasma (\mu mol/L)</th>
<th>Liver (\mu mol/g)</th>
</tr>
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<tbody>
<tr>
<td>8 d</td>
<td>SFO (n = 2)</td>
<td>0.93; 0.96</td>
<td>96.5; 84.0</td>
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<td></td>
<td>RP (150 RE) (n = 2)</td>
<td>1.06; 0.98</td>
<td>251.8; 302.4</td>
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<td>\approx 12 d</td>
<td>SFO (n = 7)</td>
<td>0.92 ± 0.05</td>
<td>151.9 ± 6.7</td>
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<td></td>
<td>RP (37.5 RE) (n = 3)</td>
<td>1.11 ± 0.09</td>
<td>215.8 ± 14.2 (^7)</td>
</tr>
<tr>
<td></td>
<td>RP (150 RE) (n = 7)</td>
<td>1.06 ± 0.09</td>
<td>298.8 ± 16.6 (^5)</td>
</tr>
<tr>
<td>15 d</td>
<td>SFO (n = 2)</td>
<td>0.94; 1.17</td>
<td>139.7; 157.9</td>
</tr>
<tr>
<td></td>
<td>RP (150 RE) (n = 2)</td>
<td>0.85; 0.92</td>
<td>340.5; 354.7</td>
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\(^1\) SFO, safflower oil (15 \(\mu L\)); RP, retinyl palmitate; RE, \(\mu g\) retinol equivalents. Rats were given either 150 or 37.5 RE or 15 \(\mu L\) SFO as a control at the ages indicated.

\(^2\) Individual values for treatment groups in which \(n = 2\); \(\bar{x} ± S.E.M\) when \(n \geq 3\).

\(^3\) Significantly different from SFO at \(\approx 12\) d (Mann-Whitney \(U\) test); \(^4\) \(P < 0.02\); \(^5\) \(P < 0.001\).

\(^6\) Significantly different from 37.5 RE at \(\approx 12\) d, \(P < 0.03\).

The increase of antigen-specific B cell clones into mature plasma cells that secrete low-affinity antibodies, mainly of the IgM class, during the primary antibody response. However, some antigen-specific naive B and T cells also differentiate into memory B and T cells on initial stimulation with antigen (22). Thus, the primary response may include antibody production and secretion as well as a “silent” response in which memory is generated. Memory B and T cells are long-lived and can be activated on restimulation with antigen to proliferate and produce the secondary antibody response, which, in comparison with the primary response, is more rapid, of greater magnitude, and of longer duration, and is characterized by B cell secretion of high-affinity antibodies predominantly of the IgG class (22).

Although previous studies have explored the effects of vitamin A status on the antibody response of young rats immunized with TT when \(\approx 40–60\) d old (11, 12, 21), to our knowledge, no previous studies have examined the consequences of vitamin A supplementation concurrent with primary immunization on the antibody response of rats vaccinated soon after birth. Therefore, in the present studies, the antibody response to TT was evaluated in vitamin A–sufficient rats immunized as nurslings and again as young adults. We also explored the consequence of vitamin A supplementation concurrent with early primary TT immunization on immunologic memory by assessing the secondary anti-TT IgG response. A previous examination of the ontogeny of the antibody response in rats from near birth to adulthood revealed that 12-d-old rats immunized with sheep red blood cells (SRBC) produced a barely detectable primary anti-IgM plaque-forming cell response, whereas 20-d-old rats produced a response that was six- to sevenfold greater (23). Thus, we initially questioned whether nursling rats primed soon after birth with TT (a T cell–dependent antigen, as is SRBC) could produce a measurable primary anti-TT response, or could form immunologic memory as evidenced by increased anti-TT IgG production after reimmunization in young adulthood. Although no primary plasma anti-TT response was detectable, all rats produced a secondary-type anti-TT IgG response when they were reimmunized as young adults, providing evidence that they had been successfully vaccinated.

\(^7\) Mann-Whitney \(U\) test; \(P < 0.001\).
as nurslings. The above results suggest that the primary response of vitamin A–sufficient nursing rats was undetectable because only a low number of naive B cells differentiated into mature plasma B cells secreting TT-specific IgM or IgG antibodies. However, the clonal expansion and differentiation of naive B and T cells along the memory pathway must have occurred during the primary response of vitamin A–sufficient rats immunized soon after birth since measurable anti-TT IgG responses were produced after reimmunization in young adulthood. Therefore, on the basis of these observations, we conclude that vitamin A–sufficient rats primed with TT at very early ages form immunologic memory to TT during the primary response, accounting for their ability to produce a secondary response after reimmunization in young adulthood.

To examine the effects of a vitamin A supplement given with early primary immunization on the primary and secondary anti-TT IgG responses, nursing rats were treated with a single oral dose of either 37.5 or 150 RE retinyl palmitate. These doses of vitamin A were chosen to be comparable with either 6250 or 30,000 RE in a 5-kg child and, therefore, to resemble vitamin A doses that are currently delivered with DPT immunization to infants and children ≤ 1 y old living in regions of endemic vitamin A deficiency (13). In the present studies, both doses of retinyl palmitate were effective in improving vitamin A status of young rats as assessed by increased hepatic storage, and neither dose produced signs of toxicity. However, there was no effect of vitamin A given concurrent with primary immunization on the secondary anti-TT response of nursing, vitamin A–sufficient rats.

These results contrast with those of Semb et al (10), who reported that high-dose vitamin A supplementation enhanced the primary anti-TT response of children aged 3–6 y old with and without ocular manifestations of mild vitamin A deficiency. There are several plausible explanations for the apparent incongruency between the results of the two studies. It is possible that the dose of vitamin A provided in the present studies was not sufficient to cause an effect on the immune system, although hepatic vitamin A stores measured 2 d after treatment were clearly elevated. It is also possible that an effect of vitamin A on the anti-TT response might have been seen in nursing rats of low vitamin A status, as has been demonstrated in older rats (11, 12, 21) and in preschool children such as those evaluated by Semb et al (10). We chose, however, to examine the effect of providing retinyl palmitate to young rats nursed by vitamin A–sufficient dams because vitamin A deficiency is very rare in the neonatal period and, therefore, it is to be expected that, in programs of vitamin A supplementation linked to vaccination programs (beginning with DPT vaccination at ~6 wk of age), a large proportion of infants will be of normal vitamin A status. If this is the case and if our results are indicative of the anti-TT response of human infants, then the effect of vitamin A supplementation on the antibody response of infants may differ significantly from that of children who are first immunized in their preschool years. Thus, it may not be appropriate to extrapolate the results from studies in preschool children to what may be seen in infants. Our results with TT immunization suggest that it may be important to specifically examine whether vitamin A has a similar effect on the immune responses of very young children immunized when ~6 wk old and of older children who receive a primary or booster immunization at 3–6 y of age.

Previous studies in neonatal mice had indicated that mice made tolerant to allogeneic skin grafts began to reject them when they were fed a normal diet supplemented with a high amount of retinyl acetate (16, 17); this result suggests that immune responses could be modulated very early in life by vitamin A. Because previous work had shown a high proportion of CD8+ T cells compared with CD4+ T cells in nursling (5–16 d old) rats (18), we were concerned that, if vitamin A activates T cells and the predominant type of T cell has a suppressive function, retinyl palmitate treatment might actually favor the suppression of T cell–dependent antibody responses in very young rats. However, the doses of retinyl palmitate that we administered to nursing rats, concurrent with early primary immunization, had no observable effect on their secondary response when rats were reimmunized as young adults. As described in Results, our analysis had sufficient power to detect a change, and thus we interpret this negative result as a true lack of effect of supplemental vitamin A on the development of immunologic memory to TT in nursling rats.

The magnitude of the secondary anti-TT IgG response was, however, clearly affected by the age of nursling rats when first immunized. A difference of just 1 wk (8 d compared with 15 d) in the ages of nursling rats at the time of priming resulted in a difference of about six- to eightfold in the secondary anti-TT IgG response (Figure 2A). These results indicate, first, that memory to this T cell–dependent antigen is formed as early as 8 d after birth in rats and, second, that the capacity to produce memory develops rapidly postnatally. A similar phenomenon has also been noted in neonatal mice immunized with mumps virus vaccine (24). This study indicated that the secondary response to mumps virus was about twofold lower in mice primed when 10 d old than when 14 d old. The above data, combined with those of the present study, suggest that the age-related differences in the secondary response of vitamin A–sufficient and retinyl palmitate–supplemented rats could reflect maturation, phenotypical, or numerical differences in B and/or T cells at the time of the primary immunization.

In conclusion, vitamin A–sufficient rats initially immunized with TT at an early age did not form a plasma antibody response, but did form immunologic memory to TT that was activated on restimulation with TT at a later age. The capacity to produce immunologic memory to TT developed significantly within the span of a week (8–15 d of age) in nursling rats. The provision of a vitamin A supplement to nursing rats from vitamin A–sufficient mothers at the time of the first immunization had neither a stimulatory nor a deleterious effect on the secondary anti-TT responses, but significantly increased hepatic vitamin A stores. We speculate that, in human infants, treatment with supplemental vitamin A concurrent with immunization would not be deleterious to their antibody response to T cell–dependent antigens.

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REFERENCES