

THE IMPACT OF VITAMIN A DEFICIENCY AND REPLETION WITH RETINOL AND β -CAROTENE ON CONCAVALIN A-INDUCED LYMPHOCYTE PROLIFERATION

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ABSTRACT

Concanavalin A (Con A)-induced splenic lymphocyte proliferation was studied in young rats during vitamin A deficiency and after oral repletion with 1500 retinol equivalents (R.E.) of either retinyl palmitate (RP) or β C-carotene (β C). Initial studies, designed to optimize the proliferation assay, showed that the response of vitamin A-deficient rats was consistently delayed in comparison to control rats fed a vitamin A-adequate diet. The overall magnitude of the proliferative response in vitamin A-deficient rats was also somewhat reduced (~34% less than that of the control group). After vitamin A-deficient rats were repleted with RP, the Con A-induced proliferative response of splenic lymphocytes resembled that of the control group in both magnitude and kinetics. However, in vitamin A-deficient rats repleted with β C, the delayed response to Con A persisted in some animals and the overall response was intermediate between that of vitamin A-deficient rats and either control rats or rats repleted with RP.

KEY WORDS: Beta-carotene, Concanavalin A, Mitogen, Vitamin A deficiency, Splenocytes, Thymidine incorporation

INTRODUCTION

Vitamin A status is thought to have important consequences for both humoral and cellular immune responses (1,2). Since the first report of mitogenic activity of phytohemagglutinin (PHA) on human leukocytes in 1960 (3), the lymphocyte proliferation assay has become a widely used in vitro test to evaluate cellular immune function in animals and humans (4,5). It is now appreciated that mitogens bind to receptors in the plasma membrane (6), initiating the intracellular signal transduction pathways that lead to mitosis and cell division. Transformation from the quiescent state to mitosis can be assessed sensitively and quantitatively by cellular uptake of the DNA precursor, [³H]thymidine (7). Particular mitogens are relatively specific for particular types of cells (5). For example, the mitogens Con A and PHA provide information on the response of T lymphocytes, lipopolysaccharide on the response of B lymphocytes, and pokeweed mitogen on the T cell-dependent response of B

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lymphocytes.

Lymphocyte transformation has been used as a test of cellular immunocompetence during vitamin A deficiency (8-15), following vitamin A repletion (8,12), and in the normal animal after supplementation with retinoids and carotenoids (13,14,16,17). Vitamin A deficiency has been associated both with a decreased (8-12,14,18) and an increased transformation response (9,13). Nauss et al. (9) showed that differences within studies may depend on the tissue source of lymphocytes (spleen, or regional lymph nodes) and on the stage of vitamin A deficiency. Differences among studies may also be related to the use by different investigators of various animal models and experimental protocols. However, the transformation response to T cell mitogens has been nearly consistently depressed during vitamin A deficiency. A decreased response to Con A and/or PHA has been reported for spleen cells (8,9,18) and lymphocytes (10) of vitamin A-deficient rats or chicks (14). Normal transformation responses were restored within 3 days after in vivo repletion with vitamin A (8).

Vitamin A deficiency also has been shown to be associated with a marked decrease in the antibody response to T cell-dependent antigens and some T cell-independent antigens [see ref. 19 and 2 for reviews]. We reported previously that the antibody response to pneumococcal polysaccharide (SSS-III, the capsular polysaccharide antigen of *Streptococcus pneumoniae*, type III) was very low in vitamin A-deficient rats as compared to vitamin A-sufficient pair-fed controls (19-21). However, the antibody response was normal when rats were repleted with RP near the time of immunization (19,20,22). The present study was designed to determine whether preformed vitamin A (RP) and provitamin A, β C, are effective in restoring the cellular immune response in the vitamin A-deficient rat. A particular strength of this study is the direct comparison of these results on lymphocyte proliferation to results on antibody production in the same animals which we have reported previously (23). The comparison of vitamin A to β C was of interest for two main reasons. First, the lack of toxicity of β C makes it an appealing form of vitamin A for human administration and, therefore, further studies of its actions with regard to the immune system are desirable. Second, supplementation studies with β C in humans (24) or with β C or the non-vitamin A precursor, canthaxanthin, in the rat (16) indicated a potential for T cell activation by non-vitamin A precursor carotenoids. These results suggested that carotenoids might have immunostimulatory properties outside of their action as a precursor of retinol. Given the reasonably consistent reduction in the proliferative response to Con A by spleen cells of vitamin A-deficient rats, we have elected to use this assay system to evaluate the ability of nutritionally equivalent amounts of vitamin A, as RP or β C, to restore cellular immune function in the previously vitamin A-deficient rat.

MATERIALS AND METHODS

Chemicals and reagents: Iscove's Modified Dulbecco Medium (IMDM), HAM's F-12 mixture, serum supplements and chemicals used to supplement the culture medium (described below), concanavalin A (Con A) and thymidine were purchased either from GIBCO (Grand Island Biological Co., NY) or Sigma Chemical Co. (St. Louis, MO). Serum supplements used in the experiments described below were: 10% fetal calf serum (FCS); 10% horse serum; a mixture of 1% controlled process serum replacement (CPSR-2, Sigma) plus 0.3% normal rat serum (NRS); we found that it was necessary to use NRS to optimize the viability of cells during the time period of the assay. Media also contained 1.3% or NRS or 1.3% vitamin A-deficient rat serum (DRS). NRS and DRS were collected from litter mates of the rats used in these experiments. These sera

were then heat-treated at 56°C for 30 min prior to use to inactivate complement. [3H]Thymidine (specific activity 20 Ci/mmol) was purchased from New England Nuclear (Dupont, Wilmington, DE). RP was obtained from Sigma and β C from either Sigma or Hoffmann-La Roche, Inc., NJ.

Animals and diets: The animals and dietary protocols used in these studies have been described in detail previously (23). Briefly, virus antibody-free female Lewis rats, housed in individual cages, were fed a purified diet free of vitamin A (A- diet) during pregnancy and until 20 d after giving birth. When the pups were 21 d-old, they were weaned onto the A- diet and assigned to the different experimental groups described below. Rats belonging to the control group received the same semisynthetic diet containing 4 μ g retinol (as RP)/g of diet.

Experiments: Preliminary experiments were conducted to optimize the experimental conditions for determining lymphocyte proliferation. Unless otherwise noted, rats were approximately 50 d-old. The main study used cells from rats that have been described previously for their antibody response to SSS-III and their tissue vitamin A concentrations [Experiment 1 in ref. (23), see Table I for summary]. Briefly, rats were divided into six groups at weaning: five groups were fed the A- diet for the next 21 d to produce vitamin A deficiency. The sixth group (control) was fed the vitamin A-adequate diet. Four of the five groups fed the A- diet were then repleted with an oral dose of either RP or β C. Five d prior to the collection of spleen cells, all the groups were immunized with 15 μ g of SSS-III (20,23). Con A-induced lymphocyte proliferation responses were tested for four of the six groups (23) in the study. These were: the vitamin A-sufficient control group (vehicle repletion only), the vitamin A-deficient group (vehicle repletion only), and two previously vitamin A-deficient groups that were repleted, respectively, with 1500 retinol equivalents (RE) of either RP or β C.

TABLE 1.

Experimental Design and Repletion Schedule for Main Study

Group	Total repletion dose (RE)*	Age (d) when:		
		orally repleted	immunized with SSS-III #	assayed for lymphocyte proliferation
A-			45	50
RP	1500	41,43,45	45	50
β C	1500	41,43,45	45	50
A+ control			45	50

* One retinol equivalent (RE) equals 1 μ g of retinol or 6 μ g of β C. Sunflower oil, 180 μ l, was used as the vehicle.

Reference 23 contains the experimental results concerning antibody production and tissue retinol levels.

Preparation of splenic lymphocytes: Splenic lymphocytes were prepared aseptically by gentle dissociation of individual spleens in Hanks Balanced Salt Solution (HBSS) using a 7-ml hand-held tissue homogenizer (Ten Broeck type) (20). The

homogenate was centrifuged at 1,500 rpm for 10 min and the cells were resuspended in a 50:50 mixture of culture medium consisting of IMDM and HAM's F-12 medium, supplemented with L-glutamine (2 mM), non-essential amino acids (1 mM), sodium pyruvate (1 mM), β -mercaptoethanol (10 mM), penicillin and streptomycin (10,000 U/ml), and sodium bicarbonate (0.5 ml of 7.5% solution per 100 ml). The specific serum supplement used is given in the legend of the figure or table describing each experiment.

Lymphocyte proliferation assay: After counting (Coulter model ZM cell counter, Coulter Electronics Inc., Edison, NJ), the spleen cells were resuspended at 5×10^6 cells/ml for stimulation by Con A which was added at a final concentration of 1.25-6.25 μ g/ml. The assay was carried out in flat-bottomed, sterile 96-well tissue culture plates (Linbro, Flow Laboratories, McLean, VA). Each test well received 100 μ l of spleen cell suspension and 100 μ l of mitogen solution; the control wells received 100 μ l of culture medium without mitogen. The culture plates were incubated in a 5% CO₂ atmosphere at 37°C for 24-120 h. Four h prior to harvesting, 25 μ l of medium containing 1 μ Ci [³H]thymidine, at 10^{-5} M final concentration unless indicated otherwise, was added to each well. The cells were harvested using a Mini-MASH-II automatic cell harvester (Whittaker M.A. Bioproducts, Walkersville, MD) onto glass fiber filters. The amount of incorporated tritium was measured by a liquid scintillation counting at an efficiency of ~33%. In all experiments, cell viability was routinely assessed by trypan blue exclusion and was found to be >98%.

The [³H]thymidine incorporation data have been expressed as corrected counts per min (ccpm), calculated by subtracting the mean cpm of the individual sample's control cultures (without mitogen) from the mean cpm of each test culture. Each ccpm value reported was a mean of at least quadruplicate determinations for each sample. To estimate the area under each proliferation curve, Simpson's rule (summation of trapezoids) was used.

Statistical analysis: All data are expressed as mean \pm SEM. Statistical analyses were performed by standard methods: Student's *t*-test for comparison of responses between two groups and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for comparison among more than two groups. Differences with $p \leq 0.05$ are reported as statistically significant.

RESULTS

Preliminary studies

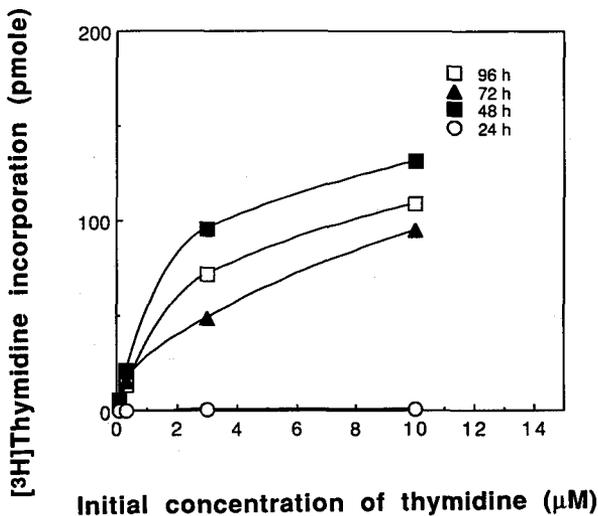
The first objective of this work was to establish optimal conditions for assaying lymphocyte proliferation for application to a nutritional study. Of particular concern were the effects of serum used to culture cells and whether the kinetics of response were the same or different for cells from normal and vitamin A-deficient rats. Thus, an initial set of four experiments was conducted to determine appropriate conditions for measuring the proliferative response of splenic lymphocytes. Thymidine concentration was evaluated, as were the dose response to Con A and the effects of various serum supplements on the rate of proliferation of cells of rats fed the vitamin A-deficient and -sufficient diets.

Thymidine concentration: We first determined the effect of thymidine concentration in the culture medium on the rate of incorporation of tritium into the

splenocytes of normal male rats (FIG. 1). The mass of thymidine incorporated, calculated from tritium content and the specific activity of [^3H]thymidine added to the culture medium, increased as the initial thymidine concentration increased from ~80 nM (labeled [^3H]thymidine without addition of non-radioactive "cold" thymidine), to 0.1, 3 and 10 μM total thymidine. Similar trends were observed after 48, 72 and 96 h of incubation with Con A. These results indicated that the endogenous thymidine pool does not support a maximal rate of thymidine incorporation. In subsequent studies, the culture medium used for labeling contained thymidine at a final concentration of 10 μM .

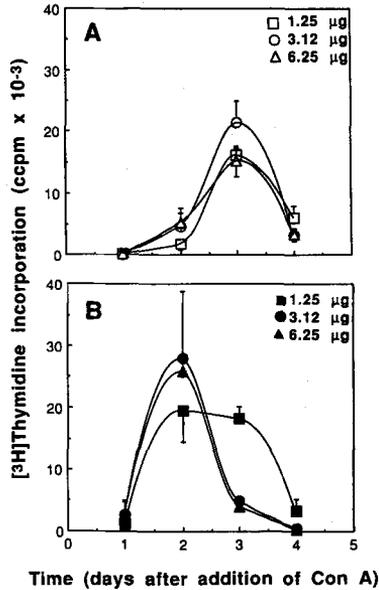
Con A stimulation: The dose response for Con A-induced lymphocyte proliferation in rats fed the control diet and vitamin A-deficient diet is shown in FIG. 2. The proliferation of cells from control rats was maximal after 48 h for Con A doses of 3.12 and 6.25 $\mu\text{g}/\text{ml}$. After stimulation with only 1.25 $\mu\text{g}/\text{ml}$ of Con A, the magnitude of response was slightly lower and peak responses were noted on both day 2 and day 3. For vitamin A-deficient rats (panel A), the maximum response occurred ~72 h after stimulation with all doses of Con A. The magnitude of the maximum response was also ~17-41% lower for cells from vitamin A-deficient rats than for cells from control rats. Thus, this study provided preliminary evidence for a shift in the kinetics of response of lymphocytes from vitamin A-deficient rats.

FIG. 1: Effect of Medium Thymidine Concentration on the Incorporation of Thymidine in Spleen Cells



Cells from three normal male rats, ~60 d-old, were examined separately under each incubation condition. Points show the mean response after 24, 48, 72, and 96 h of incubation with 1.25 $\mu\text{g}/\text{ml}$ of Con A.

FIG. 2: Thymidine Incorporation into Spleen Cells as a Function of Con A Concentration Used for Stimulation

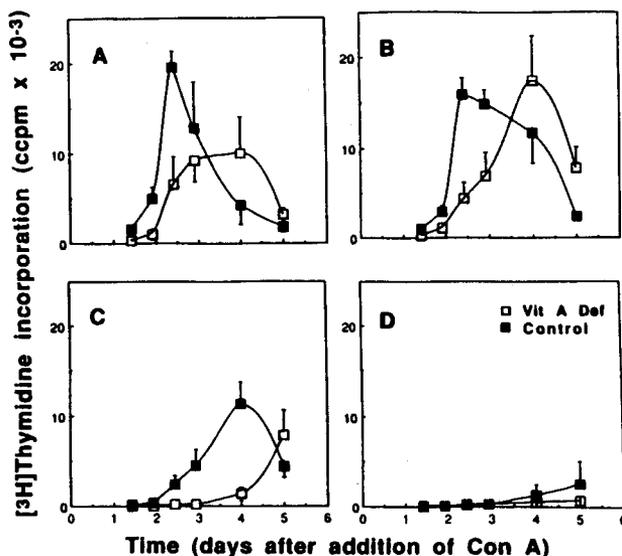


Panel A: Vitamin A-deficient rats, n=2; Panel B: Control rats, n=3.

Serum component of the incubation medium: The effects of three different types of serum supplements in the culture medium was investigated for Con A-induced proliferation of spleen cells of vitamin A-sufficient and -deficient rats (FIG. 3). The basic culture medium was supplemented with either 10% FCS, 1% CPRS-2 plus 0.3% NRS, 10% horse serum, or no supplement. The presence and type of serum in the culture medium influenced the kinetics of the proliferation response significantly. Cells from control rats showed maximal $[^3\text{H}]$ thymidine incorporation after ~30 h in the presence of 10% FCS or 1% CPRS-2/0.3% NRS, while their response in 10% horse serum was lower in magnitude and gave peak responses at a later time. In the absence of serum, $[^3\text{H}]$ thymidine incorporation was very low. Because the use of 1% CPRS-2/0.3% rat serum allowed us greater control over the composition of the incubation medium, we chose this supplement for subsequent experiments. This study also confirmed that spleen cells from vitamin A-deficient rats developed a later maximal proliferative response as compared to cells from control rats. The delay of ~36-48 h was observed irrespective of the type of serum in the culture medium.

To determine whether the small amount of retinol in the rat serum supplement would affect the kinetics or magnitude of $[^3\text{H}]$ thymidine incorporation, cells from vitamin A-deficient rats were incubated with medium containing 1.3% serum from either normal rats or vitamin A-deficient rats. After 48, 72 and 96 h of incubation, there was no significant difference in $[^3\text{H}]$ thymidine incorporation due to the source of serum.

FIG. 3: Comparison of Four Culture Media on the Proliferative Response to Con A



The media were fetal calf serum (FCS, Panel A), CPSR-II plus NRS (panel B), horse serum (Panel C) and no serum (panel D). Spleen cells were from control rats (n=3) or vitamin A-deficient rats (n=3) were incubated with 2 µg/ml of Con A for the times shown. Bars show the SEM.

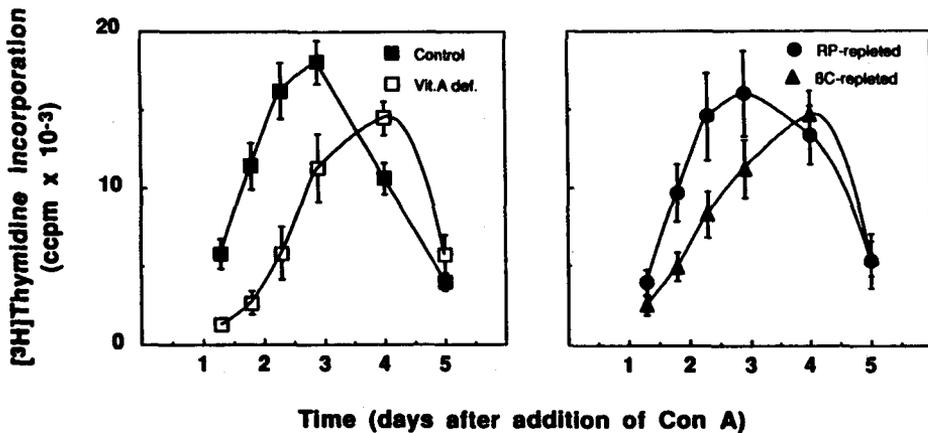
Effects of vitamin A-deficiency and repletion with retinol or β-carotene on spleen cell proliferation

Our second principal goal was to examine the effects of repleting vitamin A-deficient rats in vivo with either retinol (as RP) or βC on the in vitro lymphocyte proliferative response, and to correlate this to antibody production which was also determined in the same animals (23). FIG. 4 shows the Con A-induced [3H]thymidine incorporation in cells from 50 d-old male rats fed a vitamin A-free diet from the time of weaning. The kinetics of thymidine incorporation were followed over a 6-d period in cells from individual rats in four treatment groups: vitamin A-sufficient (control), vitamin A-deficient, vitamin A-deficient but repleted with 1500 R.E. of RP and vitamin A-deficient but repleted with 1500 R.E. of βC. Significant differences ($p \leq 0.01$) in thymidine incorporation were observed between the cells of control and vitamin A-deficient rats after 31, 44 and 55 h of incubation. The maximum response of cells from vitamin A-deficient rats was delayed by an average of 24 h relative to controls. Cells from rats repleted with RP showed a response pattern similar to cells from control rats, and also differed significantly from cells of vitamin A-deficient rats at 44 and 55 h ($p \leq 0.01$ or 0.05). Cells from rats repleted with βC showed an intermediate response. Here, the overall response pattern resembled that of cells from vitamin A-deficient rats: both showed a maximum response after 4 d and the βC group also differed significantly

from the control group after 31, 44 and 55 h of incubation ($p \leq 0.01$ or 0.05). The differences between the β C group and the RP group, however, were not statistically significant.

The total area under each thymidine incorporation–time curve was determined. Compared to cells from control rats, the integrated response of vitamin A-deficient rats was 66%, while those of RP-repleted and β C-repleted rats were 96% and 68%, respectively.

FIG. 4 Kinetics of Thymidine Incorporation into Spleen Cells of Control, Vitamin A-Deficient, RP-Repleted and β C-Repleted Rats



Lymphocytes of 50 d-old male Lewis rats were stimulated with Con A ($2 \mu\text{g}/\text{ml}$) for 38–120 h. Rats in this study were part of experiment 1A described previously (23). Rats repleted with RP received 1500 R.E. (equal to $1500 \mu\text{g}$ retinol) orally, divided into three doses and given 4, 2, and 0 d before immunization. Rats repleted with β C also received 1500 R.E. [based on 1 R.E. = $6 \mu\text{g}$ β C (25)] on the same schedule. Incubation medium contained 1% CPSR-2 + 0.3% NRS as the serum supplement. Values shown are the mean \pm SEM of 8 rats per group.

In a follow-up study of more limited scope, cells from vitamin A-deficient, RP-repleted and β C-repleted were compared over a 96-h time course. Again, the response of cells from 5 vitamin A-deficient rats was delayed (maximum at 72 h) as compared to cells from 3 rats repleted with RP (maximum at 48 h; $p < 0.01$). In this study, the response of spleen cells of rats repleted with β C more closely resembled that of the RP-repleted group and also differed significantly ($p < 0.01$ at 48 h) from that of vitamin A-deficient rats.

DISCUSSION

A number of studies have used the *in vitro* lymphocyte proliferation assay to investigate the ability of retinoids or carotenoids to restore function in previously vitamin A-deficient animals or to stimulate cells from animals with a normal vitamin A status. A variety of animal models have been studied [rat (8-11,15,16,18), chick (11,13,14) and cow (26)]. With one exception (15), the literature is consistent that the transformation of spleen cells stimulated with T-cell mitogens is reduced during vitamin A deficiency (8,9,18). The results of the present study shed additional light on the differences in response between the vitamin A-deficient and the normal animal and provide new comparative data on the efficacy of RP and β C to restore the proliferative response in the previously vitamin A-deficient rat. Additionally, it is possible to relate these results on lymphocyte proliferation directly with the antibody response studied in the same animals.

Preliminary studies showed that the concentration of thymidine provided by a typical culture medium and high-specific activity [3 H]thymidine did not support maximum thymidine incorporation. Beyer and Bowers (27) have previously discussed the importance of using a medium thymidine concentration that does not limit the synthesis of nucleic acid. The lymphocyte proliferation assay has most often been used to compare the response of well-defined cell populations after various treatments *in vitro*. In this type of study, it would seem reasonable to assume that the endogenous thymidine pool in all cells is equal. However, when this technique is applied to studies of individuals or treatment groups in which the endogenous pools might differ and endogenous thymidine could be rate-limiting, then the addition of saturating exogenous thymidine is important for true comparisons of the maximum proliferative potential among treatments. Although the 3 H cpm incorporated into spleen cells were lower when cold thymidine was added, the actual mass of incorporated thymidine increased (FIG. 1). Thus, subsequent experiments were conducted with 10 μ M thymidine, higher than concentrations used in most previous studies.

It is typical to determine the time of maximum thymidine incorporation after mitogen stimulation in a preliminary experiment and to use this time to evaluate responses in subsequent comparative studies (28-31). In our experience, the time of maximum thymidine incorporation, even for control cells, has differed somewhat from experiment to experiment, despite consistent use of the same medium, serum supplements and our attempts to control other experimental variables (32). Given this seemingly inherent variability, which has been noted previously (7), together with the preliminary observation that the kinetics of response of control and vitamin A-deficient rats differed, we believed it essential to conduct complete time studies for individual animals in order to correctly evaluate any differences in lymphocyte transformation between dietary groups. When we used this methodology, the response of cells from vitamin A-deficient rats was, indeed, consistently low at the time when the response of cells from vitamin A-sufficient rats was maximal (FIG. 2, 3, 4). Had only this time point been examined, the results might have been interpreted as a generally reduced proliferative response. However, the response of cells from vitamin A-deficient rats continued to increase and reached a maximum at a later time than did control cells. This delay was similar to that recently reported by Friedman et al. (33) in which peripheral blood leukocytes from vitamin A-deficient chicks developed a slower proliferative response to heat-killed *E. coli* than cells from vitamin A-sufficient chicks. The reasons for such delays are not understood. The observation suggests that there is not simply a lower fraction of responsive cells in the spleen cell population of the

vitamin A-deficient animal but, rather or in addition, that the signal transduction event(s) leading to the proliferative response is defective. On average, the integrated response of cells from vitamin A-deficient rats was 66% of control (FIG. 4). Thus, these studies have revealed that there are both *quantitative and qualitative* differences in lymphocyte proliferation in the vitamin A-deficient state.

It seemed possible that vitamin A-deficient cells might undergo a "repair" during culture in medium supplemented with 10% FCS (~10-15 ng retinol /ml medium). However, when cells were cultured with less serum (1.3% rat serum) the cells of vitamin A-deficient rats still developed a proliferative response and there was no significant difference between vitamin A-deficient cells cultured in medium containing DRS or NRS. Further kinetic studies with addition of physiological concentrations of various retinoids or carotenoids to the culture medium could provide insight into the role of retinoids, if any, in the *in vitro* proliferative response of these cells.

The second objective of this study was to compare the ability of RP vs. β C, administered in nutritionally equivalent amounts *in vivo*, to restore immune function in terms of lymphocyte proliferation and antibody response in the vitamin A-deficient rat. Pneumococcal polysaccharide lacks polyclonal activating properties and thus immunization with SSS-III was not expected to affect the lymphocyte proliferative response measured 5 days later. In support of this, the magnitude of the proliferative response was similar in these rats and in non-immunized rats used in preliminary studies. As previously reported (23), the antibody response to pneumococcal polysaccharide (SSS-III) after rats were repleted with RP equalled 141% of control for the SSS-III-specific PFC response and 96% of control for plasma anti-SSS-III. These values were both significantly different from the responses of vitamin A-deficient rats (4% and 25% of control for SSS-III-specific PFC and plasma anti-SSS-III, respectively), indicating a restoration of ability to respond to antigen. In the present study, [³H]thymidine incorporation by spleen cells of RP-repleted animals was normal in terms of magnitude and kinetics of response, indicating that the ability of T lymphocytes to respond to Con A was also restored. However, for rats repleted with β C, the results were less consistent. The antibody response to SSS-III was essentially normal after β C repletion (158% of control for the SSS-III-specific PFC response and 95% of control for plasma anti-SSS-III, respectively) (23). Yet, in the same animals, the lymphocyte transformation response of spleen cells to Con A was still delayed, resembling the response pattern of vitamin A-deficient rats. In other experiments, the response of some β C-repleted animals approached that of rats repleted with RP. Thus, it should not be concluded that β C was consistently effective or ineffective, but rather that it functioned less reliably in the restoration of the cell-mediated immune response than did RP. The reason for this difference is not known but could be related to heterogeneity among T cells. Although SSS-III is considered a T cell-independent antigen (34,35), the antibody response to SSS-III is known to be regulated through the action of T-amplifier and T-suppressor cells (34,36,37). It is possible to speculate that, as compared to those cells stimulated to proliferate by Con A, the T lymphocytes involved in antibody regulation either were not limiting for the antibody response, or recovered more quickly after RP or β C repletion through either cellular repair mechanisms or through maturation from precursor or stem cells.

Finally, although a role for carotenoids in immunostimulation has been proposed, there was no indication in our studies that β C is more effective than preformed vitamin A in restoring immune functions in the previously vitamin A-deficient animal.

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