

## **Impaired T lymphocyte immune response in vitamin A depleted rats and chicks**

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**Vitamin A deficiency results in decreased immune responses;** the objective of the present study was to investigate the involvement of T lymphocytes in the depression of immune responses resulting from vitamin A depletion. This objective was achieved by evaluating antigen-specific T lymphocyte proliferative responses *in vitro* as vitamin A depletion developed. The evaluation was performed in both rat and chick to examine the generality of immune effects due to vitamin A depletion. Our findings show that vitamin A depletion led to severe impairment of T lymphocyte activity in both animal models, and that this was directly related to the vitamin A status in both species. Immune response impairment was found to precede other manifestations of vitamin A deficiency, and was rapidly corrected by feeding retinyl acetate boluses. This implied a possible regulatory, rather than constitutive, role of vitamin A in immune responsiveness.

### **Immune response: Retinol: T lymphocytes: Chick: Rat**

Vitamin A possesses a multiplicity of physiological functions, in particular in vision, reproduction, maintenance of epithelial tissues and growth (Sklan, 1987). The molecular mechanism of action of vitamin A has only been described in vision (Wolf, 1984).

Vitamin A deficiency is also associated with depressed immunity (for review, see Shapiro & Edelson, 1985). This phenomenon has been described in various *in vivo* and *in vitro* immunological assays (Cohen & Cohen, 1973; Dennert *et al.* 1979; Lotan & Dennert, 1979; Barnett & Bryant, 1980; Malkovsky *et al.* 1983): vitamin A deficiency is accompanied by low levels of serum immunoglobulins (Harmon *et al.* 1963; Leutskaya & Fais, 1977; Sirisinha *et al.* 1980), impaired IgG (Barnett, 1983; Smith & Hayes, 1987) and IgA responses (Sirisinha *et al.* 1980), reduction in delayed-type hypersensitivity (DTH) reactions (Uhr *et al.* 1963; Athanassiades, 1981; Smith *et al.* 1987), depressed responses to mitogens (Nauss *et al.* 1979; Davis & Sell, 1983) and reduced natural killer-cell activity (Nauss & Newberne, 1985). Conversely, supplementary vitamin A has been reported to enhance immune responses (Shapiro & Edelson, 1985).

These studies raise three topics to be addressed in the present study. The first refers to the immune cell type that is affected by vitamin A. It appears that the impairment of immunoglobulin synthesis in vitamin A deficiency is limited to IgG and IgA synthesis and not to IgM (Barnett, 1983; Smith & Hayes, 1987). This finding may indicate that the main effect of vitamin A on the immune response is mediated through the activity of T lymphocytes, a notion supported by reports on reduced responses to mitogens in deficiency (Nauss *et al.* 1979). However, the limitations of these mitogen studies result from the fact that the responses of T lymphocytes are polyclonal and not antigen-specific, and that these responses do not describe functional aspects of T lymphocyte subpopulations (Sharon, 1983). The second point is that there is no information available describing the time-scale of the relationship between vitamin A deficiency and the onset of immune depression. The

Table 1. *Composition of diet (g/kg)*

Ingredient	Composition
Soya-bean meal (450 g protein/kg)	350
Sorghum	588
Soya-bean oil	20
Choline chloride	2.2
Methionine	0.7
Sodium chloride	2
Calcium carbonate	10
Dicalcium phosphate	15
Mineral mix*	10
Vitamin mix†	2

\* The mineral mix contained (mg/kg diet):  $\text{FeSO}_4$  2.5,  $\text{CoCO}_3$  0.64,  $\text{KI}$  3.8,  $\text{MnSO}_4$  208,  $\text{Na}_2\text{MoSO}_4$  3.5,  $\text{CuSO}_4$  20;  $\text{ZnSO}_4$  220; inert filler to 1 kg.

† The vitamin mix contained (mg/kg diet): thiamin 5, riboflavin 5, nicotinic acid 60, pantothenic acid 15, pyridoxine 5, folic acid 4, biotin 0.2, menaquinone 0.5, cyanocobalamin 0.02,  $\alpha$ -tocopheryl acetate 60, cholecalciferol 0.05; inert filler to 1 kg.

only information available describes a static state of immune responsiveness at different stages of vitamin A deficiency (Nauss *et al.* 1985). The third point refers to the universality of the immune effects due to vitamin A deficiency. Most studies on the impairment of the immune response resulting from vitamin A deficiency were carried out in mammals, usually in rats, with only a few addressing general immunological variables in the chick, such as antibody synthesis, lymphoid organ structure, or leucocyte numbers (Bang *et al.* 1972, 1973; Leutskaya & Fais, 1977; Davis & Sell, 1983). To our knowledge, there is no information on the effects of vitamin A deficiency on T lymphocyte antigen-specific responses in birds.

The main objective of the present study was, therefore, to study the time-scale of the effect of vitamin A depletion on the antigen-specific response of T lymphocytes of the helper phenotype in both the rat and chick.

## MATERIALS AND METHODS

### *Animals*

Male Lewis rats were bred and raised in our own breeding colony. The rats were maintained on commercial feed purchased from the Weizmann Institute of Science Animal Breeding Center until 4 weeks of age, and then transferred to the experimental diets (Table 1; Sklan *et al.* 1987) with or without 1 mg retinol equivalent/kg, in an environment-controlled facility with free access to water and feed. Male chicks of a commercial strain 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 diets (Table 1; Sklan *et al.* 1987), which were either with or without 1 mg retinol equivalent/kg. Animals were weighed at 15 or 7 d intervals for rats and chicks respectively. In some experiments, both vitamin A-depleted and control rats were given two separate boluses of 30 mg retinyl acetate (Sigma, St Louis, MO, USA)/kg; the boluses were administered by mouth 3 and 5 d after immunization.

### *Determination of retinol*

Retinol and retinyl esters were determined by reverse-phase high-performance liquid chromatography on a C<sub>18</sub> column using retinyl acetate as an internal standard (Sklan & Halevy, 1984).

### *Antigens and immunizations*

Ovalbumin (chicken egg; OVA) and bovine serum albumin (BSA) (both grade V) were purchased from Sigma (St Louis, MO, USA). Incomplete Freund's adjuvant (IFA) and Complete Freund's adjuvant containing 1 mg *Mycobacterium tuberculosis* H37Ra/ml (CFA) were purchased from DIFCO (Detroit, MI, USA). Purified protein derivative (PPD) of mycobacteria was purchased from Statens (Denmark). *M. tuberculosis* par. avium and its PPD were provided by the Central Veterinary Laboratory (Surrey, UK). Rats were immunized as follows: each rat received 50 µg OVA emulsified in CFA in two injections, 50 µl each, delivered to the two hind foot pads. Chicks were immunized as follows: each chick received 1 mg BSA emulsified in IFA containing 2 mg ground *M. tuberculosis* par. avium organisms/ml, injected into multiple subcutaneous sites (a total of 1 ml).

### *T lymphocyte proliferation assay*

The in vitro assay for antigen-specific proliferation of T lymphocytes has been described (Friedman & Cohen, 1983). In brief, draining popliteal lymph node lymphocytes (in the rat) or peripheral blood lymphocytes (in the chicken), were prepared in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with antibiotics, 1 mM glutamine, non-essential amino acids (10 g/l), Na-pyruvate (10 g/l) (all from Bio-lab, Jerusalem), 1 × 10<sup>-5</sup> M 2-mercaptoethanol (Eastman-Kodak, Rochester, NY, USA) and either normal rat serum (10 ml/l) or normal chicken serum (40 ml/l).

Lymphocytes were cultured in ninety-six well cluster plates (Costar, Cambridge, MA, USA) in 200 µl medium (rat 5 × 10<sup>5</sup> cells/well, chick 1 × 10<sup>6</sup> cells/well) in the presence or absence of antigen: OVA 5 µg/well, PPD 5 µg/well (which assayed the response of the animals to the respective mycobacterial antigens present in the adjuvants). The cultures were placed in a humidified incubator containing carbon dioxide in air (75 ml/l) at 37.5° or 40° for rat and chick cells respectively. Following 92 h of culture, each well was pulsed with 1 µCi [<sup>3</sup>H]thymidine (specific activity 10 Ci/mmol; Nuclear Research Center, Negev) for 4 h. The cultures were then harvested onto filters by a multiharvester (Dynatech, Wesbart, UK), and counted in a liquid-scintillation counter. The results of individual animals are the average of quadruplicate cultures and are expressed in counts/min (cpm).

### *Assay of antibody production*

Antibodies specific for OVA and BSA were detected in sera of rats or chicks respectively by means of a solid-phase radioimmunoassay (RIA; Friedman & Cohen, 1983) or an enzyme-linked immunosorbent assay (ELISA; Bartlett *et al.* 1976). In brief, dilutions of rat or chick sera were added to microtitre plates coated with OVA or BSA respectively. After extensive washes to remove excess unbound antibody, the bound rat anti-OVA antibodies were determined in a RIA by using a <sup>125</sup>I-labelled goat anti-rat immunoglobulin (Amersham International, Amersham, UK), and the bound chick anti-BSA antibodies were determined in an ELISA using a peroxidase-rabbit anti-chicken immunoglobulin (Bio Makor, Rehovot). Non-specific binding was blocked by either rat or chick serum which had previously been shown to have no specific antibody activity. The results of individual animals are the average of triplicate measurements and are expressed in maximal antibody titres (the reciprocal or the log<sub>5</sub> of the maximal serum dilution).

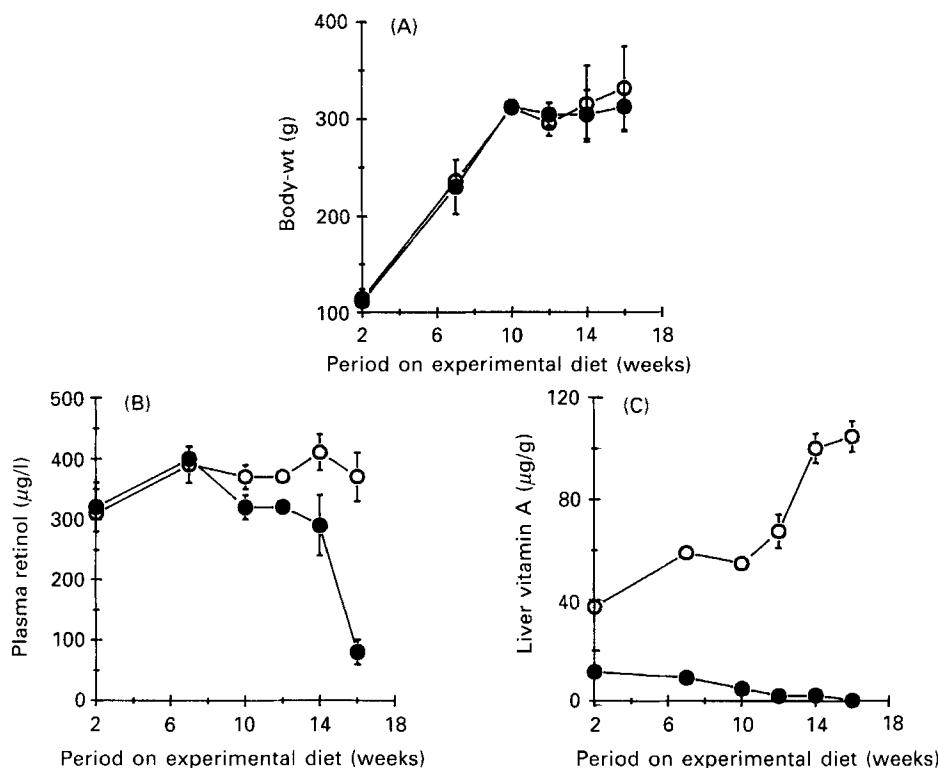


Fig. 1. Vitamin A status in rats receiving experimental diets with (○) or without (●) 1 mg retinol equivalent/kg. The rats were transferred to the experimental diets on weaning, and the first measurements were taken 2 weeks later; for details of diet and procedures, see Table 1 and p. 441. Points are mean values, with their standard errors represented by vertical bars when they do not fall within the area of the symbol, of three individual rats. (A) Body weight, (B) plasma retinol, (C) liver vitamin A.

#### Statistical analyses

Significance of differences were determined using Student's *t* test.

#### RESULTS

The vitamin A status, as reflected by liver and blood vitamin A levels in rat and chick is presented together with body-weights in Figs 1 and 2 respectively. Liver vitamin A levels of the rat were reduced after 2 weeks on the experimental diets, and decreased to levels of 6 μg/g within 7 weeks (Fig. 1(C)); after this time, a reduction in circulating retinol became evident (Fig. 1(B)). In the chick, depletion occurred more rapidly both in liver and in blood (Fig. 2(C and B respectively)). Body-weights of vitamin A-depleted animals did not differ from those of their normal controls in the time period tested and feed intakes (not shown) did not differ (Figs. 1(A) and 2(A)).

All immunological studies reported here were performed within the periods shown in Figs 1 and 2, 16 weeks in the rat (i.e. 20 weeks of age) and 35 d (7 weeks) in the chick, giving an insight into changes in immune responsiveness as vitamin A depletion progressed. The first immunological assays were performed 2 weeks after the animals were put on the respective diets.

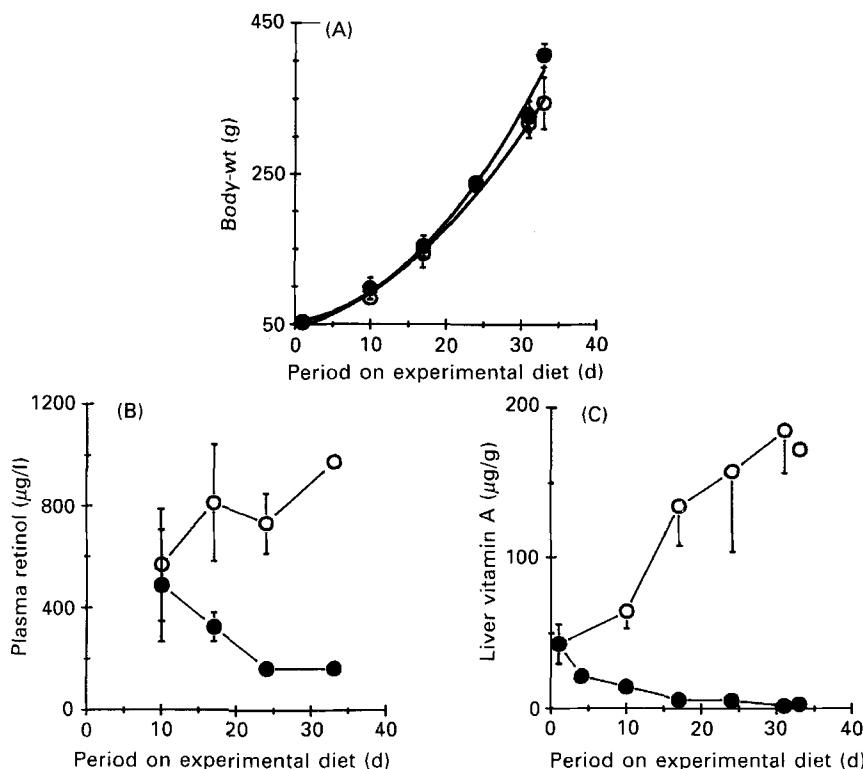


Fig. 2. Vitamin A status in chicks receiving experimental diets with (○) or without (●) 1 mg retinol equivalent/kg. The chicks were put on experimental diets on hatching; for details of diet and procedures, see Table 1 and p. 441. Points are mean values, with their standard errors represented by vertical bars when they do not fall within the area of the symbol, of three individual chicks. (A) Body-weight, (B) plasma retinol, (C) liver vitamin A.

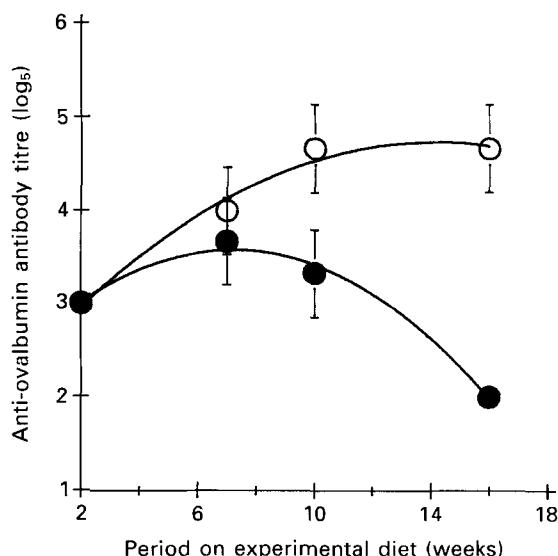


Fig. 3. Serum antibody response of vitamin A-depleted rats (●) and controls (○) to ovalbumin (OVA). Rats were immunized with 50  $\mu\text{g}$  OVA emulsified in complete Freund's adjuvant in the hind footpads. After 10 d serum was prepared, and assayed in a solid-phase radioimmunoassay for antibodies to OVA; the results are expressed as the log<sub>5</sub> dilution of sera that gave significant binding above background levels. For details of experimental procedures, see p. 441. Points are mean values, with their standard errors represented by vertical bars when they do not fall within the area of the symbol, of three individual rats.

Table 2. Serum antibody responses of vitamin-A depleted chicks to bovine serum albumin (BSA)†  
(Mean values with their standard errors for three chicks)

Day of experiment	Mean reciprocal antibody titre			
	Vitamin A-depleted		Control	
	Mean	SE	Mean	SE
17	6000*	3000	21000	7500
22	16000*	11000	53000	15000

Values were significantly different from respective control values: \*  $P < 0.05$ .

† Chicks were immunized with 1 mg BSA emulsified in incomplete Freund's adjuvant containing 2 mg *Mycobacterium tuberculosis* par. avium/ml. After 10 d, sera were assayed by an enzyme-linked immunosorbent assay for antibodies reactive against BSA. The results are the reciprocals of the last serum dilution whose reaction was significantly above background levels.

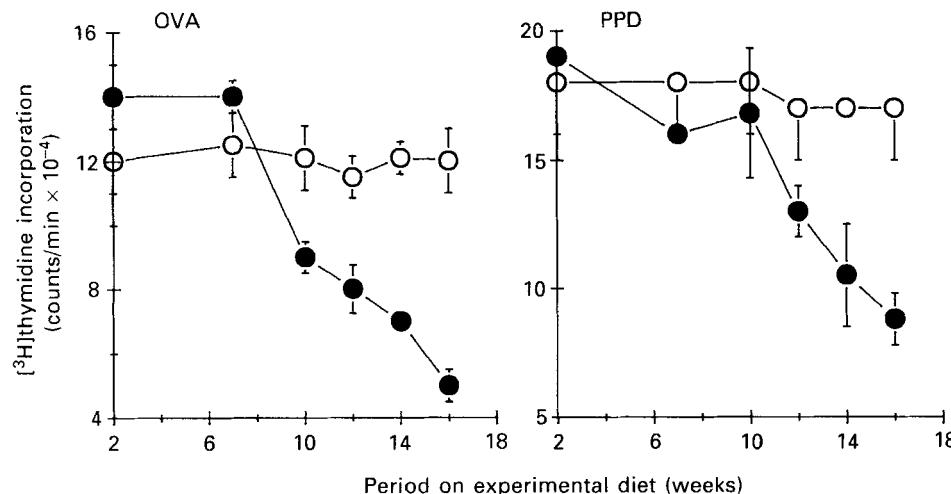


Fig. 4. T lymphocyte proliferative response of vitamin A-depleted rats (●) and controls (○) to two protein antigens. Rats were immunized with 50 µg ovalbumin (OVA) emulsified in complete Freund's adjuvant in the hind footpads. After 10 d popliteal lymph node cells were prepared and assayed for their proliferative response to either OVA or purified protein derivative (PPD). The degree of proliferation was determined by  $[^3\text{H}]$ thymidine incorporation. For details of procedures, see p. 441. Points are mean values, with their standard errors represented by vertical bars when they do not fall within the area of the symbol, of quadruplicate cultures of three individual rats.

Rats and chicks were immunized with OVA and BSA respectively, emulsified in adjuvant. Blood was withdrawn 5–10 d later and anti-OVA-BSA antibodies were determined. (The time points appearing in Figs and Tables refer to time of assay after commencement of diets.) As can be seen (Fig. 3) the anti-OVA specific humoral immune response of vitamin A-depleted rats was severely reduced from week 10. This coincided with the rapid depletion of vitamin A (Fig. 1). A similar decrease was observed in the BSA-specific antibody response of the chick (Table 2). The decrease in the antibody response of Vitamin A-depleted chicks was observed on day 17, which was earlier than in the rat. As in the rat, this period coincided with rapid vitamin A depletion (Fig. 2). The time between

Table 3. *T lymphocyte proliferative response of vitamin A-depleted chicks to purified protein derivative (PPD)†*

(Mean values with their standard errors for quadruplicate cultures from three individual chicks expressed in counts/min)

Day of experiment	[ <sup>3</sup> H]thymidine incorporation (counts/min)			
	Vitamin A-depleted		Control	
	Mean	SE	Mean	SE
20	9857*	2919	71192	16962
35	2858*	1408	64623	9274

Values were significantly different from respective control values: \*  $P < 0.05$ .

† Chicks were immunized with incomplete Freund's adjuvant containing 2 mg *Mycobacterium tuberculosis* par. avium/ml. After 10 d peripheral blood lymphocytes were assayed for their proliferative response to PPD. The degree of proliferation was determined by [<sup>3</sup>H]thymidine incorporation.

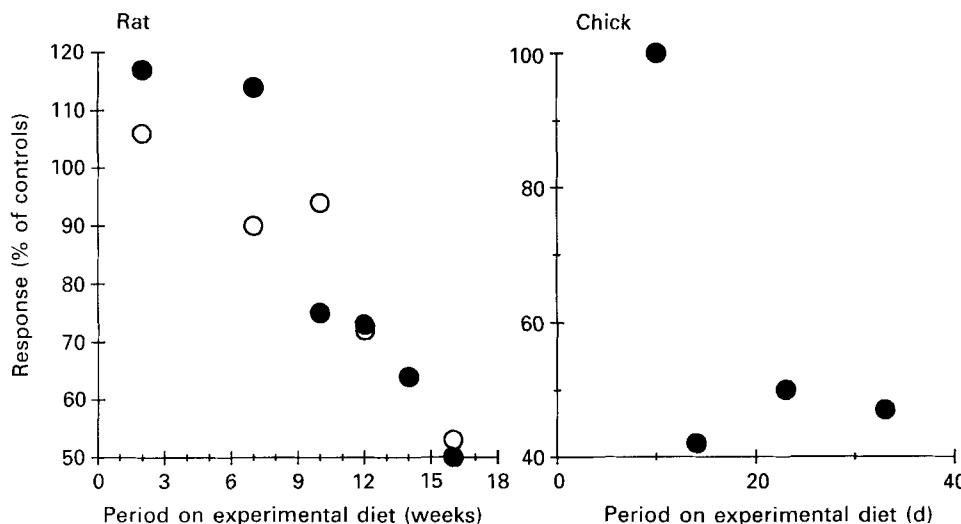


Fig. 5. T lymphocyte proliferative response in vitamin A-depleted rats and chicks. The proliferative response of vitamin A-deficient rats (ovalbumin, OVA – ● and purified protein derivative, PPD – ○) and chicks (PPD) (points are mean values for three individual animals) is expressed as percentage response of the respective controls. For details of experimental procedures, see p. 441.

immunization and serum preparation had no effect on these observations (values not shown).

The lowered specific antibody response could have been the result of the defective activities of B lymphocytes, helper T lymphocytes, or both. To ascertain the involvement of T lymphocytes in defective immune responses resulting from vitamin A depletion, we evaluated the antigen-specific, *in vitro*, proliferative response of T lymphocytes. Rats and chicks were immunized with OVA emulsified in CFA, or IFA containing 2 mg *M. avum*/ml, respectively. Antigen-specific proliferative response assays were performed 7–9 d later. In these assays several doses of antigen were routinely used (1–25 µg/well, with identical results) to eliminate the possibility of altered kinetic responses; optimal responses

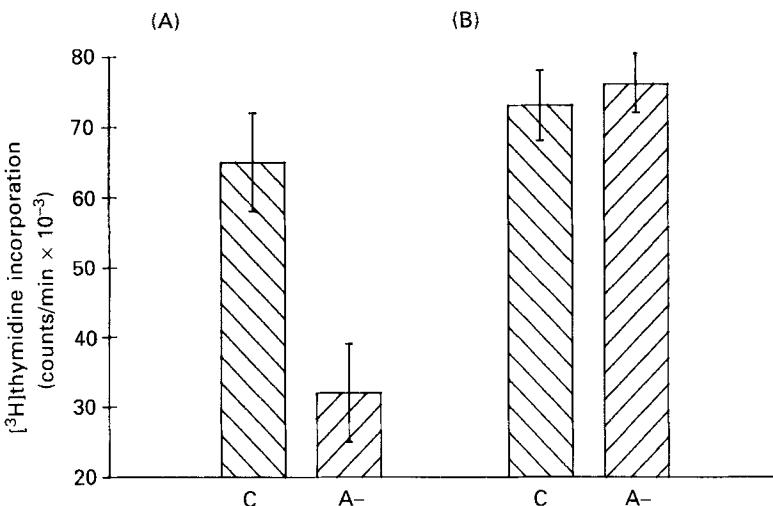


Fig. 6. Restoration of immune competence in vitamin A-depleted rats following vitamin A repletion. Vitamin A-depleted and control rats were immunized with 50 µg ovalbumin (OVA) emulsified in complete Freund's adjuvant. After 3 and 5 d rats were given two separate boluses of 30 mg retinyl acetate/kg. At 4 d after the last bolus, popliteal lymph node cells were assayed for their proliferative response to OVA. The degree of proliferation was determined by  $[^3\text{H}]$ thymidine incorporation, and results are mean values, with their standard errors represented by vertical bars when they do not fall within the area of the symbol, of four individual rats. (A) Responses of groups not given boluses, (B) groups given boluses. C, Controls; A-, vitamin A-depleted rats.

were noted at 5 µg antigen/well. Antigen-specific T lymphocyte proliferative responses of both vitamin A-depleted rats and chicks were found to be reduced (Fig. 4 and Table 3). The decreased responses in the vitamin A-depleted rat were demonstrated for two antigens, OVA and PPD; the time-course over which the reduced responses developed was similar for both antigens.

The deterioration of the T lymphocyte immune response is further demonstrated by another experiment (Fig. 5), which expresses the T lymphocyte proliferative response of vitamin A-depleted animals as a percentage of their normal controls. This means of normalization was chosen in order to standardize non-specific, *in vitro*-caused fluctuations in the responses of control and experimental animals. As shown, the development of the decreased T lymphocyte response to both PPD and OVA in the rat (Fig. 5(A)) occurred after a period of 10 weeks, and decreased to 50% of the control response; the development of defective T lymphocyte responses in the chick (Fig. 5(B)) was more dramatic, occurring within 17 d, and was approximately 40% of the control's response to PPD. In both cases the impairment of the immune response was not total, but was severely suppressed.

To demonstrate further the relationship between vitamin A and immune competence, OVA-immunized, vitamin A-depleted rats and control rats were fed with two separate doses of retinyl acetate (each of 30 mg/kg), administered 3 and 5 d after immunization. The anti-OVA T lymphocyte proliferative response was evaluated 4 d after the final bolus; repletion of vitamin A resulted in rapid recovery of the T lymphocyte response of vitamin A-depleted rats to levels equalling those of controls treated in the same manner (Fig. 6). Identical results were observed in the chick (values not shown).

#### DISCUSSION

Vitamin A deficiency has been shown to be associated with depressed immune responses (Shapiro & Edelson, 1985). Studies supporting this observation have described immune depression in man and rodents, and have also reported suppression of various *in vivo* immune responses (Dennert, 1984). The present study extends previously reported findings in two important aspects: (1) we present results showing vitamin A-linked immune suppression in the fowl and, for the first time, we demonstrate defective *in vitro* antigen-specific T lymphocyte responses, and (2) we show these defective responses to be directly related to vitamin A status in both fowl and rat. These findings were demonstrated in a state of vitamin A depletion, a stage that precedes that of deficiency (Nauss *et al.* 1985; Smith *et al.* 1987).

The effect of vitamin A deficiency on the impairment of immunoglobulin production has been widely reported in various mammals (Shapiro & Edelson, 1985; Dennert, 1984), whereas the relevant information in the chicken is limited (Panda & Combs, 1963; Leutskaya & Fais, 1977). The present report confirms previous findings in the mammal and extends them under similar conditions to the fowl. These results differ from those reported by Davis & Sell (1983), who did not detect any reduction in antibody production in vitamin A-deficient chicks. This discrepancy is probably due to the difference in immunization protocols and to the much less sensitive passive haemagglutination used by these investigators compared with the sensitive ELISA used in the present study. When comparing antibody levels between rat and chick it was interesting to note that the impairment of antibody production in the chick developed sooner than in the mammalian counterpart (Fig. 1, Table 1). This observation was confirmed by the T lymphocyte assays, and may be due to the larger vitamin A reservoirs present in the rat following suckling.

The precise mechanism by which vitamin A deficiency or depletion affects immunoglobulin production is not known; however, the effect could be systemic, not specifically immune response related, or could be immune system specific (Shapiro & Edelson, 1985). Thus, vitamin A could have a direct effect on the metabolic capacity of B lymphocytes to differentiate into plasma cells or, alternatively, have a direct effect on immunoglobulin production by plasma cells. However, as recently reported by Smith & Hayes (1987), B lymphocyte differentiation and plasma cell production of IgM were not impaired in vitamin A-deficient mice, thereby implying intact B lymphocyte physiology. On the other hand, these investigators demonstrated a severe impairment of IgG production, indicating the possible involvement of T lymphocytes as possible targets for the immune-response-related effects of vitamin A deficiency. This concept is supported by previous studies investigating *in vivo* T lymphocyte-dependent immune responses, such as DTH (Uhr *et al.* 1963; Athanassiades, 1981; Smith *et al.* 1987). However, a direct *in vitro* evaluation of antigen-specific T lymphocyte functions in vitamin A-deficiency cases has not been attempted, with the possible exception of T lymphocyte responses to mitogen stimuli (Davis & Sell, 1983; Malkovsky *et al.* 1983; Nauss *et al.* 1985); these, however, are polyclonal, and not functional (Sharon, 1983). Our main objective herein was to evaluate antigen-specific T lymphocyte responses in vitamin A depletion in both mammal and bird. As we have shown, vitamin A depletion caused approximately 50% depression of the *in vitro* antigen-specific T lymphocyte proliferative response after 15 weeks in the rat and 15 d in the chick. The onset of immune response impairment appeared to precede other physiological manifestations of vitamin A deficiency, such as loss of body-weight, eye lesions, retarded growth, ataxia and hypoxia (Davis & Sell, 1983; Smith *et al.* 1987; Sklan, 1987).

The direct effect of vitamin A on T lymphocyte function was also demonstrated in repletion experiments: the impairment of the immune response in vitamin A-depleted rats

was rapidly restored to control levels by vitamin A repletion, thereby indicating the direct effect of retinol on T lymphocyte antigen-specific responses. This rapid *in vivo* rehabilitation of immune responsiveness was surprising, though not unexpected, for the addition of retinoic acid to *in vitro* cultured macrophages was reported to produce immunological related effects within several hours (Moore *et al.* 1984; Wirth & Kierszenbaum, 1986). These observations imply that the involvement of vitamin A in immune responsiveness might be regulatory or functional rather than constitutive (Smith *et al.* 1987).

The T lymphocytes proliferating *in vitro* have been shown to be of the initiator-helper phenotype (Friedman *et al.* 1983; Maron *et al.* 1983). We propose, therefore, that IgG production impairment in vitamin A-deficient rodents (Smith & Hayes, 1987) could be a direct result of helper T lymphocyte malfunction (Malkovsky *et al.* 1983), although defective B-cell function has not been ruled out. This malfunction might be explained by either a direct effect of vitamin A or its analogues on T lymphocyte function, such as IL-2 production, which is increased following vitamin A supplementation (Colizzi & Malkovsky, 1985), or by an indirect effect resulting from a macrophage-related defect in antigen presentation, Ia expression or IL-1 production (Goldman, 1984, 1985; Shapiro & Edelson, 1985; Wirth & Kierszenbaum, 1986). These various possibilities are currently being explored.

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