HUMORAL AND CELL-MEDIATED IMMUNITY IN VITAMIN A-DEFICIENT LAMBS

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Animal Science

(ABSTRACT)

Humoral and cell-mediated immunity were monitered in control (Con), vitamin A-deficient (A-def) and vitamin A-repleted (A-rep) lambs. Humoral immunity was assessed by measuring antigen-specific and polyclonal immunoglobulin G (IgG) concentrations. Cell-mediated immunity was assessed by measuring in vitro peripheral blood lymphocyte proliferation in response to optimal and suboptimal concentrations of concanavalin A (ConA), phytohemaglutinin (PHA) and the antigens ovalbumin, lysozyme and human gamma globulin. Polyclonal serum IgG concentrations were unaffected by treatment in Trial I while in Trial II concentrations were greater ($P < .05$) in the A-def lambs during the HGG challenge period. Antigen-specific IgG concentrations in both trials tended to be greater in the Con lambs towards the end of both the ovalbumin (Trial I and II) and lysozyme (Trial I) challenge periods. Control and A-rep lambs in Trial I responded similarly to the HGG challenges. In Trial II both the A-def and A-rep lambs had

lower (P<.10) HGG specific serum IgG concentrations on the last 3 wk of the HGG challenge period. Unstimulated lymphocyte proliferation in Trial I was greater (P < .08) in the A-def lambs prior to repletion and numerically greater in the A-rep lambs after repletion but not different between treatments in Trial II. Lymphocyte proliferation stimulated by an optimal concentration of ConA was not different between treatments in Trial I but was greater in the A-def lambs during the HGG challenge period in Trial II. Less than optimal concentrations of ConA elicited proliferations that were lower (P < .07) in the A-rep lambs in Trial I and higher in the A-def lambs (P < .07) (HGG challenge) in Trial II. Proliferations in response to optimal PHA concentrations were unaffected by treatment in Trial I but tended to be greater during the HGG challenge in the A-def lambs in Trial II. Suboptimal PHA stimulated lymphocyte proliferation was lower in the A-def lambs in Trial I (P < .06) and higher (P < .09) in the A-def lambs in Trial II (HGG challenge period). Antigen-stimulated lymphocyte proliferation was not different between treatments in Trial I. In Trial II ovalbumin stimulated lymphocyte proliferation was not different between treatments but the A-def lambs tended to have greater lymphocyte proliferation in response to HGG.

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Chapter I

Introduction

Vitamin A, one of the earliest vitamins to be discovered, has eluded attempts to uncover its function in metabolism. Vitamin A's role in vision, though important, cannot be this vitamin's only function since an animal dies from a vitamin A deficiency but not necessarily from blindness. Many other physiological functions have been associated with vitamin A but the biochemical basis of these functions is not well understood. One such function associated with vitamin A which has received much interest in the past decade involves vitamin A's effects on the immune system.

Vitamin A-deficient animals are more susceptible to bacterial, protozoal, viral and rickettsial infections (Rogers, 1970). Epidemiologic studies have also linked low dietary vitamin A intake to an increased risk of lung cancer (Shekelle, 1981). Some researchers have reported that vitamin A-deficient animals exhibit decreased antibody titers in response to an immunogenic challenge (Krishnan et al., 1974) while others report decreased lymphocyte proliferation in response to various mitogens (Nauss et al., 1979). Many theories have been hypothesized to explain

these occurrences but the mechanisms behind them are still not known. One possible theory not yet explored involves the relationship between vitamin A, glucocorticoids and the effect of glucocorticoids on the immune system. Researchers are still not in agreement on vitamin A's effects on serum glucocorticoid levels. Some investigations point to elevated levels of serum glucocorticoids in vitamin A-deficient animals while others show no relationship between vitamin A-deficiency and glucocorticoids. Watson et al. (1986) reported a decrease in serum cortisol concentrations in humans supplemented with 13-cis retinoic acid, a synthetic retinoid. It is well established, however, that elevated glucocorticoid levels have detrimental effects on the immune system.

The task undertaken in this project, therefore, was to monitor humoral and cell-mediated immunity in control, vitamin A-deficient and vitamin A-repleted lambs in an attempt to determine the effect of vitamin A status on immune function. Humoral immunity was monitored by measuring antigen-specific and polyclonal serum immunoglobulin G (IgG) concentrations. Cell-mediated immunity was monitored by measuring peripheral blood lymphocyte proliferation in response to both optimal and

suboptimal concentrations of concanavalin A (ConA), phytohemaglutinin (PHA) and specific antigens. Serum cortisol concentrations were also monitored throughout the study.

Chapter II

Literature Review

Immune System. The immune system of an animal is a complex network of specialized cells acting together, either directly or through soluble factors in response to substances which are foreign to the body.

The first resistant force encountered by a foreign substance or antigen (Ag) is the external defense system. This system includes such things as skin, epithelial linings of the gastrointestinal tract, respiratory tract and genitourinary tract, mucus, lysozyme, tears and many others (Barrett, 1980).

Once an Ag penetrates this first line of defense it may activate a whole network of events. Phagocytic cells such as polymorphonuclear leukocytes, monocytes, macrophages and others may engulf the Ag and destroy it. Antigen-presenting cells (APC) can also process the Ag and present antigenic determinants to B and T lymphocytes. In the APC the Ag is altered or enzymatically cleaved to yield peptide fragments that contain the antigenic determinants of the processed Ag. The antigenic determinants (epitopes) then become associated with the class II major

histocompatability (MHC) molecules at the surface of the APC. The complex between the epitope and the class II MHC molecule is necessary for recognition by a helper T-cell (Sell, 1987).

Binding of the T-cell Ag receptor to the class II epitope complex on the APC begins the process of T-cell activation. The APC then releases a lymphokine called interleukin 1 (IL-1). Interleukin 1 induces the T-cell to expose surface receptors for the lymphokine interleukin 2 (IL-2). The T-cell then releases IL-2 which serves to stimulate T-cells carrying the IL-2 receptor to proliferate (Sell, 1987).

The activated T-helper cell can then convey "help" to Ag-activated B-cells. The "help" is given through soluble factors (lymphokines) which cause the B-cell to divide and differentiate into an antibody-producing plasma cell. B-cells may be stimulated to produce antibodies without the help of T-cells but the Ag must be of a special type and the antibodies produced are primarily IgM. Re-exposure of a B-cell to a T-cell independent Ag leads to the same level of antibody production seen in the first exposure. In other words, T-independent Ag do not give rise to memory (Myrvik and Weiser, 1984).

Activated T-cells release an array of lymphokines with a variety of functions. Macrophage chemotactic factor acts

on monocytes and macrophages causing them to migrate to the site of T-cell response. Macrophage activation factor(s) (MAF) stimulates the macrophage to become more active in the process of ingestion and killing of bacteria, tumor cells and other foreign substances. The action of MAF is probably the result of gamma interferon which is a component of the MAF lymphokines (Roitt, 1980).

T-helper cells are not the only subclass of T-cells. Cytotoxic T-cells (CTL) are involved in the recognition and lysis of virally infected cells. These T-cells recognize Ag in the context of class I MHC molecules. Interleukin 2 stimulates CTL precursors to proliferate and mature. Following IL-2 stimulation, CTL can kill virally infected target cells. The CTL do not die after the lysis of a virally infected cell but can seek out and continue to kill other infected target cells (Barrett, 1983).

The immune system is composed of checks and balances necessary to protect an animal from foreign substances without recognizing and reacting against ones own tissues. Suppressor T-cells (Ts) function in a manner needed to keep the immune system from "overreacting". Suppressor T-cells can suppress T-helper (Th) functions, B-cell functions and CTL functions as well. Recent evidence suggests that Ts are actually made up of a group of cells with varying surface markers and functions (Asherson et al. 1986).

B-cells are a second major type of lymphocyte and are primarily responsible for humoral immunity. B-cells express Ag-specific immunoglobulins (Ig) on their cell surface. B-cells whose surface Ig bind to the appropriate Ag and receive help from Th cells are triggered to differentiate. A portion of the B-cells will proliferate and differentiate into plasma cells and a portion will transform back to resting mature B-cells called memory cells. Plasma cells are specialized antibody-producing cells. The Ab produced by the plasma cell have the same specificity as the Ig found on the precursor cell. The Ab produced can serve such functions as toxin neutralization, coating of specific Ag for increased phagocytosis, complement activation and others.

This overview of the immune system is simplistic and superficial at best, but one can see that the defense by the body against foreign substances is complex. Questions concerning the affects of nutrition on the immune system become even more complex. A malfunction or reduction in numbers of any one of the cell types or soluble products involved in the immune system may affect many other components of the immune system as well.

Early Work. The relationship between vitamin A and the immune system was observed over a half century ago. Bloch

(1928), for instance reported that humans with vitamin A deficiency had an increase in severity and frequency of infections. Scrimshaw et al. (1968) reviewed 50 individual studies and came to the conclusion that the severity and frequency of viral, bacterial and parasitic infections are higher among vitamin A-deficient animals. Vitamin A supplementation, on the other hand, has been observed to increase resistance to Listeria Pseudomonas and Candida infections (Cohen and Elin, 1974; Hof and Emerling, 1979). These observations, however, do not confront the question of exactly how the immune system is being affected. Is the cell-mediated immune system or humoral immune system or both affected by a vitamin A deficiency? Is the affect of the vitamin A-deficiency due directly or indirectly to the vitamin A status of the animal? Does repletion of a vitamin A-deficient animal reverse the immunosuppression and if so, how much time is required before "normal" immune function returns. Several researchers in the past decade have confronted these questions.

External Defense. As has already been mentioned, the first resistant force encountered by a foreign substance is the external defense system. Vitamin A plays a critical role in maintaining the integrity of the anatomic barriers which make up this defense system (Neumann, 1977). The

earliest signs of vitamin A-deficiency include damage to the epithelial linings of the respiratory passages, digestive tract, urinary tract, eyes and buccal cavity. Keratinization, metaplasia and horny degeneration occur causing breaks in the membranes (Bogart, 1973). These breaks in the membranes may serve as entry ways for invading microorganisms. In the respiratory tract, ciliated cells which keep the membranes free of microorganisms and debris are lost and replaced by keratinized cells. The damaged epithelium also looses its ability to produce mucous secretions and with it goes the bacteriocidal activity associated with lysozyme and secretory immunoglobulin A (sIgA) (Gross and Newberne, 1980).

Increased infections of the upper and lower respiratory tracts are well documented in vitamin A-deficient states (Guthrie, 1971). Bang and Bang (1969) observed a keratinizing metaplasia of the respiratory mucosa with a concomitant enhancement of absorption of virus in damaged respiratory tissue of vitamin A-deficient chickens. The mucociliated epithelium was replaced by squamous cells. They suggested that in vitamin A-deficient populations the respiratory mucosa is especially susceptible to repeated infections. The ability to correctly interpret results from experiments involving vitamin A-deficient animals is often complicated by

variations in infection rates. Altered immune functions in vitamin A-deficient animals may be the consequence of increased infection rates and not directly to vitamin A.

Lymphoid Organs. Organs associated with the immune system can be categorized as either central or peripheral lymph organs. The central lymph organs include bone marrow, thymus and in the chicken the bursa of fabricius. Bone marrow is responsible for the formation of the precursor cells which may enter either the thymus or the bursa of fabricius. In the thymus these precursor cells differentiate into T-cells while in the bursa of fabricius they differentiate into B-cells (Hyde and Patnode, 1978).

The peripheral lymph organs include lymph nodes, spleen, tonsils and peyers patches. These organs act as filters in different areas of the body, filtering various body fluids. The peripheral lymph organs provide sites for the interactions of immune cells with Ag's, lymphocyte proliferation, cellular collaboration and antibody production (Eisen, 1980).

The effects of vitamin A-deficiency on both the central and peripheral lymph organs have been extensively studied. The results from these investigations, however, are highly variable.

Some researchers report that vitamin A-deficiency in rats has no effect on relative thymus and spleen weight (Nauss 1979, 1985). Still others report marked decreases in relative thymus weight in rats (Bang, 1973; Krishnan, 1974 ; Zile, 1979; Davis, 1983). Upon further observation of the thymus, the cortical region was found to be devoid of lymphocytes. Consistently it has been observed that vitamin A-deficient chickens have decreased thymic and bursa of fabricius weights (Nockels, 1967). After 30 d on a vitamin A-deficient diet the epithelium of the bursa of fabricius became pseudostratified and was disorganized as compared with normal tissue. Infection of vitamin A-deficient chickens with Newcastle's disease virus caused further regression of the bursa including metaplasia and keratinization (Panda and Combs, 1963). Some of the variability in these investigations may be due to the stage of the deficiency or the effects of protein energy malnutrition (PEM) which sometimes occurs along with a vitamin A deficiency. The effect of supplemental vitamin A on the size and cellularity of peripheral lymph organs has also been investigated. Moderate supplementation (250 $mg \cdot kg^{-1}$ diet) of retinal acetate to mice resulted in an enlargement of the peripheral lymph nodes (Dennert and Lotan, 1978). Supplementation of elevated levels of retinoic acid (1000 $mg \cdot d^{-1}$) was found to decrease lymphoid

organ size and cellularity. Taub et al. (1970) also observed a decrease in cellularity of peripheral lymph nodes in mice injected with vitamin A. It therefore seems that severe excesses and deficiencies of vitamin A result in changes of lymph organ size and cellularity. The magnitude of this effect differs with varying retinoid types.

Cell-Mediated Immunity. Cell-mediated immunity is that part of the immune system which is mediated by T-cells. T-cells can act as helper cells for Ab production by B-cells, as mediators of delayed type hypersensitivity (DTH) and mixed lymphocyte culture responses or they can act as cytotoxic cells to the appropriate target cell. Delayed type hypersensitivity reactions require prior sensitization by an Ag and are measured by a skin swelling reaction 24 h after Ag challenge. Dennert and Lotan (1978) found either no effect or a suppressed effect of vitamin A supplementation on DTH responses in mice depending on the dosage. In another study, vitamin A was administered with the sensitizing Ag. This protocol stimulated DTH reactions in contrast to Dennert and Lotan's (1978) observations (Athanassiades 1981). It has also been observed that cancer patients treated with vitamin A develop an increase in skin reactions to various Ag's (Micksche 1978).

Another parameter used to measure cell-mediated immunity involves the rejection of either skin or tumor grafts. It has been widely observed that skin grafts are more rapidly rejected when the mice which receive the transplantation are supplemented with vitamin A (Floersheim and Bollag, 1972; Jurin and Tannock, 1972). Large doses of vitamin A have been shown to cause rejection of autologous skin grafts (Boss, 1966; Floersheim and Bollag, 1972). Theoretically this should not happen because the immune system should not be able to recognize or respond against autologous tissue. It is therefore unclear whether vitamin A's affects on skin graft rejection are due to an enhanced cell-mediated immune system or to a direct toxic affect on the skin graft resulting in a more efficient rejection by the T-cells (Dennert, 1984).

In many tumor models, pretreatment with vitamin A prior to tumor challenge results in a lower incidence of tumors and inhibition of tumor growth. Supplementation with high doses of retinoic acid seemed to suppress cytolytic activity of spleen cells against a syngeneic tumor (tumors from identical individuals) (Glaser and Lotan 1979). In a series of experiments involving syngenic tumor models Dennert (1979) conclusively showed that vitamin A stimulated the induction of T-killer cells specific for the syngenic tumors studied.

Another method used to measure cell-mediated immunity involves the in vitro stimulation and blast transformation of T-cells by mitogenic agents. Lectins from the plants Phaseolus vulgaris (PHA) and Concanavalia ensiformis (ConA) specifically stimulate T-cells while pokeweed mitogen (PWM) stimulates both T and B-cells and lipopolysaccharide (LPS) stimulates only B-cells. All of these compounds are considered mitogens. Lymphocyte transformation can be easily assessed by measuring DNA synthesis through tritiated thymidine incorporation. The substances used to stimulate lymphocyte proliferation can be divided into two groups, nonspecific mitogens (ie ConA, PHA, LPS) and antigens. Mitogens can stimulate 60-90% of all the lymphocytes cultured regardless of their Ag specificity (Barrett, 1983). Antigens used to stimulate lymphocyte proliferation can only do so to previously sensitized lymphocytes. Lymphocytes from Ag-naive individuals fail to proliferate in vitro in the presence of that Ag.

In assessing the immunocompetence of lymphocytes from various nutritionally stressed animals it has been observed that suboptimal mitogen dosages may be of greater value. In a number of immunodeficiency states the proliferative response to optimal mitogen concentrations is normal. Only at suboptimal concentrations will the immunodeficiency be observed (Gershwin et al. 1985).

Mitogen-proliferative responses have been assessed in thymic and splenic lymphocyte populations. Dennert and Lotan (1978) observed no stimulatory effect of vitamin A on lymphocyte proliferation in vitro. Nauss et al. (1979) observed no effect of vitamin A deficiency on ConA (T-cell mitogen) responses of thymic cells. In contrast, the splenic lymphocyte response to ConA , PHA or LPS was significantly suppressed in vitamin A-deficient rats. Vitamin A supplementation for 3 d restored the mitogenic response of splenic lymphocytes to normal levels. Retinol given to cancer patients increases the mitogenic response of peripheral blood lymphocytes (Micksche et al., 1977). Slidell et al. (1981) observed that retinoic acid enhanced in vitro human tonsilar lymphocyte tritiated thymidine incorporation induced by PHA. Other researchers have found that certain retinoids at various concentrations can inhibit T-cell mitogenesis. Skinnider and Geisbrecht (1979) showed that retinol at high concentrations in vitro inhibited human T-cell proliferation to mitogens. Inhibition of human T-cell mitogenesis was also reported by Moriguchi et al. (1985) using several retinoids and carotenoids. The information on the effects of retinoids on the in vitro proliferation of lymphocytes is contradictory and unclear. Length of incubation, retinoid concentration,

type and concentration of mitogen used as well as many other factors could account for the discrepancy in results.

Not only does the effect of mitogen stimulation on peripheral lymphocytes seem to be influenced by vitamin A status, but the Ag trapping function of these lymphoid organs may also be impaired. Takagi et al. (1983) found that vitamin A-depleted rats exhibited marked deterioration in Ag-stimulated trapping of labelled cells in lymph nodes. The deterioration in Ag trapping of labelled cells was determined to be due to a derangement in the lymph nodes and not to any change in the lymphocytes.

Macrophages. Macrophages are involved in the induction of immune responses, in nonspecific resistance and in anti-tumor host defense. Any effects exerted by vitamin A on macrophages could greatly affect the responsiveness of the immune system of the animal (Rhoades and Oliver, 1980). Rats deficient in vitamin A develop parasitemia at a much faster rate than either the control or pair-fed rats (Jurin and Tannock, 1972). It was concluded that the inability of phagocytic cells to clear the infection was the reason for this observation (Krishnan et al., 1974). Supplementation with vitamin A has afforded inconclusive results. Tengerdy and Brown (1972) and Hof and Emmerling (1979) both observed enhancement of resistance due to stimulation of the

mononuclear phagocytic system. Moriguchi et al., 1985 found that gradually increasing dietary intake of retinyl palmitate increased peritoneal macrophage production of IL-1 and phagocytosis of opsonized sheep red blood cells. These elevated levels of vitamin A also activated peritoneal macrophages for the in vitro lysis of tumor target cells. Even short term exposure (4 d) to high levels of retinol, retinoic acid and retinyl palmitate induced increased tumoricidal activity of alveolar macrophages (Tachibana et al., 1984). Rhoades and Oliver (1980) on the other hand, showed that vitamin A at physiological concentrations suppressed phagocytosis. They also observed that vitamin A supplementation enhanced the production of arginase, a tumoricidal macrophage enzyme.

Humoral Immunity. The humoral immune response culminates in the production of antibodies. Additions of vitamin A along with the Ag enhance the stimulation of the humoral immune response (Dresser, 1968; Spitznagel and Allison, 1970; Jurin and Tannock, 1972; Cohen and Cohen, 1973; Leutskaya and Fais, 1977). Falchuck (1977) showed by assaying mucosal extracts that vitamin A also stimulates the local immune response (i.e. sIgA). It can be said that

vitamin A acts as an adjuvant when given concomitantly with an Ag.

Studies on the effects of vitamin A deficiency on the humoral immune response show varying results. A significant depression in antibody production to diptheria toxoid was observed by Ludovici and Axelrod (1951) and Pruzansky and Axelrod (1955). Chickens marginally deficient in vitamin A show a decrease in the production of antibodies against S. pullorum Ag. Experiments involving vitamin A-deficient weanling swine show similar results. Harmon et al.(1963) have reported a significant correlation between antibody titer and serum vitamin A concentrations in swine. Underhal and Young (1956), however, found that vitamin A-deficient mice infected with swine influenza virus showed no difference in hemagglutination titers as compared to control titers. Krishnan (1974) observed different effects of vitamin A deficiency on antibody production depending on the Ag used. Antibodies produced against sheep red blood cells (SRBC) were reduced to levels only 50% of controls in animals with combined PEM and vitamin A deficiency. This depression in antibody production was attributed primarily to PEM. Specific inhibitory effects of vitamin A deficiency were observed, however, when the animals were exposed to both diptheria and tetanus toxoid. Gershwin et al. (1984) examined the effects of vitamin A deficiency on mice

inherently susceptible to autoimmune disease (New Zealand black). They observed an increase in IgM auto-antibodies in the vitamin A-deficient mice. An increase in serum polyclonal Ig was also observed in the mice fed the vitamin A-deficient diet.

The discrepancies in results observed regarding vitamin A's effects on Ig production may be a consequence of several factors. Age, sex, species and strain of animal used in the experiments could lead to variations in results. Dosages and routes of Ag administration also affect results. A nutritional induced defect in Ab production is usually more pronounced when the Ag is of low immunogenicity or in very low dosages (Herlyn and Glaser, 1976).

The relationship between vitamin A deficiency and serum complement levels may be somewhat linked to PEM. Madjid et al. (1978) investigated the effect of PEM and vitamin A deficiency on serum complement levels separately to determine each situations effect. It was concluded that vitamin A deficiency alone has but a minor depressant effect during the early stages of the deficiency and thereafter complement levels are increased. PEM was shown to have an immediate and marked decrease in serum complement levels.

Vitamin A and Glucocorticoids. Vitamin A is necessary for tissue growth (Bieri, 1968), synthesis of specific glycoproteins (Deluca, 1984), proper development of bone (Fell, 1950), spermatogenesis (Thompson, 1964), oogenesis (Thompson, 1969), fetal and embryonic growth (Thompson, 1969) and vision (Wald, 1960). One function which has received much heated debate is vitamin A's possible role in steroidogenesis.

In a review, Johnson and Wolf (1960) stated that glucocorticoid production was inhibited in vitamin A-deficient rats. The severity of the deficiency determined which steps were blocked in adrenal glucocorticoid production. Reduced adrenal glucocorticoid production was also indicated by Glick (1963), who reported that vitamin A-deficient chicks were unable to increase blood heterophil counts in response to adrenocorticotrophic hormone (ACTH) injection. In another investigation, Nockels and Kienholz (1967) reported that marginal vitamin A deficiency blocks the normal production of glucocorticoids with the precursors being shunted to androgen producing pathways. Not all research points to a decrease in glucocorticoid production in a vitamin A-deficient state.

Perek and Kendler (1969) observed that the hyperglycemic response of their chicks to a vitamin A-

deficiency may be related to the increased activity of the adrenal cortex during this imposed nutritional stress. They also noted in an earlier study that a vitamin A deficiency impaired the bursa of Fabricius (Perek and Kendler, 1969). They concluded that this impaired growth was due to an increased adrenal concentration of steroids which are known to have lympholytic action. Gruber et al. (1976) observed increased plasma corticosterone levels in vitamin A-deficient rats. An elevated level of glucocorticoids was also suggested by Webb et al. (1969) as the reason for polyuria in vitamin A-deficient sheep.

Vitamin A deficiency is usually accompanied by PEM which affects plasma cortisol concentrations. Alleyne and Young (1966) found that children with PEM had raised levels of plasma cortisol and these raised levels depressed the thymo-lymphatic system. Schonland et al. (1972) observed that the mean plasma cortisol level in 15 children with PEM soon after admission to the hospital was significantly higher than in ten well nourished, healthy children of similar age.

Watson et al., (1986) recently observed a decrease in plasma cortisol levels of humans supplemented with 13 cis-retinoic acid. The subjects received 10mg·d^{-1} for 9 mo. By the end of the 9-mo period plasma cortisol concentrations had declined as much as 40-50%. Supplementation with other

nutrients such as vitamin E decreases plasma cortisol concentrations (Lim et al., 1981). There is a growing wealth of information suggesting nutrient status can influence plasma cortisol concentrations. The exact mechanism or extent of cortisol suppression is still under debate.

Glucocorticoids as Immunosuppressants. The immune system is an adaptive response of the body to Ag. Three principle cell types respond to Ag: macrophages, T-cells and B-cells. The interactions between these three cell types are necessary for optimum protection against most Ag. A malfunction in any one of these cell types may lead to a decrease in responsiveness to Ag. Glucocorticoid actions on the immune system extend over a wide spectrum from the killing of lymphocytes to subtle modulations of cellular interactions.

Butler and Rossen (1973) gave 17 adult, normal volunteers 96 mg of the corticosteroid methylprednisolone daily for 3 to 5 d and compared results of 12 untreated controls who were studied simultaneously. Of the treated volunteers, 86% had significant decreases in the concentrations of serum IgG. They proposed that this decrease in serum IgG concentration was due to an increase in catabolism as well as a decrease in Ig synthesis. The

latter being of most importance. Animals with high corticosterone blood levels have reduced numbers of Ig secreting cells as well as a decrease in splenic mass (Del Rey et al., 1984). Gwazdauskas et al. (1978) evaluated the relationships of antibody production to porcine red blood cells in calves and plasma glucocorticoid concentrations near the stressful time of weaning. This stressful period resulted in increased plasma glucocorticoid levels and a concomitant decrease in antibody titer.

Glucocorticoids exert their inhibiting powers on T-cells at primarily three levels. They inhibit IL-1 and IL-2 production and may also decrease proliferation of T-cells and thus reduce the population of T-cells that produce immune interferon (Munck et al., 1984). These effects can account in large part for the suppression by glucocorticoids of cell mediated immunity.

Interleukin 1, is a product of macrophages. Physiologically, it accounts partly for the role macrophages play in the regulation of the proliferation of T-cells since it stimulates T-cells to produce IL-2 (Oppenheim et al., 1982). Cortisol at physiological concentrations has recently been found to inhibit IL-1 production.

Interleukin 2 appears to provide the key signal for proliferation of Ag-activated T-cells, and thus is

essential for the clonal expansion that follows the initial Ag recognition phase of the normal immune response (Watson et al., 1983). The discovery that glucocorticoids inhibit IL-2 production (Gillis et al., 1979) greatly clarified the understanding of how glucocorticoids suppress primary immune responses. Subsequent experiments with cloned cells have demonstrated that there is a direct effect on IL-2 production independent of that on IL-1. Not all effects of glucocorticoids on lymphocyte proliferation are necessarily due to lack of IL-2. Proliferation of some mouse T-cell clones can be partly inhibited by dexamethasone even in the presence of excess amounts of IL-2 (Kelso and Munck, 1984).

The third principle cell type that responds to Ag is the monocyte or macrophage. Rinehart et al. (1974) observed decreased monocyte chemotaxis, decreased bactericidal activity as well as decreased phagocytosis in glucocorticoid-treated monocytes. Atkins and Frank (1974) showed that cortisone inhibited the complement independent clearance of IgG sensitized erythrocytes. They concluded that this was due to some action of glucocorticoids on the interaction between IgG on the erythrocyte surface and its receptors on splenic and hepatic macrophages. It was later determined that glucocorticoids inhibit the expression of Fc receptors on macrophages (Crabtree et al., 1979). The Fc fragment of an Ig represents the carboxyl half of both

heavy chains joined by disulfied bonds as observed after papain treatment of IgG. These receptors are necessary for Ig-macrophage interactions.

Glucocorticoids may have an effect on macrophages even after phagocytosis has occurred. Merkov et al. (1971) observed that macrophages from steroid-treated animals were unable to destroy spores of Aspergillus flavus despite their ability to ingest these particles. Goldstein (1975) proposed that glucocorticoids inhibit the merger between lysosomes and phagocytic vacuoles containing ingested microorganisms thereby directly interfering with the effective killing of pathogens by phagocytic cells.

Endogenous glucocorticoids may act directly on B-cells or through internal regulators of the function of this cell, such as T-cells and accessory cells and/or their soluble products. These glucocorticoids may be a part of an autoregulatory mechanism of the immune system. This mechanism supports the concept that the circadian inverse relation between blood lymphocytes (Thompson, 1980), certain immune functions (Halberg, 1983) and the daily fluctuations of blood glucocorticoid levels is not a mere coincidence in time. In addition, the depression of certain immune functions due to elevated levels of glucocorticoids during stress (Monjan, 1982) may not be an association that occurs only in extreme situations but is rather the

expression of the above mentioned immuno-regulatory circuit operating at an elevated level. Any circumstance which may cause an elevated level of glucocorticoids could have a detrimental impact on the ability of an animal to react to Ag.

Chapter III

HUMORAL IMMUNITY IN VITAMIN A-DEFICIENT LAMBS

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ABSTRACT

Antigen-specific and polyclonal serum immunoglobulin G (IgG) concentrations were measured in control (Con), vitamin A-deficient (A-def) and vitamin A-repleted (A-rep) lambs. In Trial I ewe lambs were injected with primary and secondary antigenic challenges of ovalbumin (1 mg) and lysozyme (.1 mg). The A-def lambs were then repleted with vitamin A and all lambs were injected with primary and secondary antigenic challenges of human gamma globulin

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(HGG) (.1 mg). In Trial II Con and A-def wether lambs were given primary and secondary antigenic challenges of ovalbumin (20 ug). Half of the A-def lambs were then repleted with vitamin A. All lambs were subsequently given a primary and secondary challenge of HGG (20 ug). Spleen wt were similar for all treatments in Trial I while A-def lambs in Trial II had greater spleen wt ($P < .01$) than Con or A-rep lambs. Polyclonal serum IgG concentrations were unaffected by treatment in Trial I while in Trial II concentrations were greater ($P < .05$) in the A-def lambs during the HGG challenge period. Antigen-specific IgG concentrations in both trials tended to be greater in the Con lambs towards the end of both the ovalbumin (Trial I and II) and lysozyme (Trial I) challenge periods. Control and A-rep lambs in Trial I responded similarly to the HGG challenges. In Trial II both the A-def and A-rep lambs had lower ($P<.10$) HGG specific serum IgG concentrations on the last 3 wk of the HGG challenge period as compared to A-def lambs. Humoral immune function appears to be impaired in A-def lambs and a 2-wk repletion period was not sufficient in this study to restore humoral immune function to normal levels.

(Key words: Vitamin A, Humoral Immunity, IgG, Ovalbumin, Lysozyme, Human Gamma Globulin)

Introduction

Investigations over the last two decades have demonstrated a link between vitamin A status and immune function. Numerous studies have demonstrated that both the frequency and severity of bacterial, viral and protozoal infections are increased in vitamin A-deficient animals (Bang and Foard, 1971; Cohen and Elin, 1974; Darip et al., 1976). Infections inrease the requirement for vitamin A and thus infections and deficiencies of vitamin A aggrevate one another. Vitamin A-deficient animals have decreased splenic and thymic mass (Bang, 1973; Krishnan et al., 1976), decreased serum and secretory immunoglobulin concentrations (Ludovici and Axelrod, 1951; Sirisinha et al., 1980), decreased number and activity of macrophages (Krishnan et al., 1976) and normal serum complement levels (Madjid et al., 1978). Conversely, supplementation of vitamin A above that recommended by the National Research Council (NRC) can increase antibody production to various antigens (Sugimura et al., 1973), increase the number of antibody-producing cells in the spleen of mice immunized with sheep red blood cells (Cohen and Cohen, 1973) and decrease morbidity in bacterially challenged chicks (Tengerdy and Brown, 1977). Conversely, supplementation of vitamin A in lamb diets

above that recommended by the NRC fails to show positive effects (Nockels, 1987).

The ability to develop diets that optimize growth rate and efficiency as well as immune function could be of great economic importance in the livestock industry. Decreasing mortality and/or morbidity, especially during critical times such as birth, weaning and shipping should be of major concern. A thorough understanding of vitamin A's effects on immune function is necessary before recommendations for this nutrient can be made.

The objective of this study was to investigate the relationship between serum and liver vitamin A levels and antibody production in lambs. Antigen-specific and polyclonal immunoglobulin G (IgG) concentrations were measured to monitor humoral immune function.

Experimental Procedure

Trial I. Thirty crossbred ewe lambs weighing approximately 30 kg were blocked according to wt and randomly assigned to either a control (Con) or vitamin A-deficient (A-Def) treatment. The lambs were treated for internal parasites (levamisole[5]) and vaccinated against multiple clostridial species (Ultrabac-7[6]). The lambs were housed individually under constant lighting conditions in raised pens (.8 m x 1.0 m) with expanded metal floors and equipped with automatic nipple waterers. The lambs were fed once daily at a rate of 900g·head^{-1}·d^{-1} of a whole oat diet (Table 1). In addition to that supplied in the diet, the control lambs received a 100,000 IU oral dose of vitamin A palmitate[7] in capsule form every 2 wk.

When the mean serum vitamin A concentration of the A-def lambs fell below 20 ug·dl^{-1} the lambs were exposed to a series of antigenic challenges. The lambs were first challenged with ovalbumin. Half of a suspension containing 1 mg ovalbumin in .5 ml Freund's incomplete adjuvant and .5 ml physiologic saline was injected i.m. in the hind leg and the remainder injected s.c. along the lateral thorax.

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[5]Pitman-Moore, Washington Crossing, NJ. 08560.
[6]Beecham Laboritories, Bristol, TN. 37620.
[7]R.P. Scherer Corp., Clearwater, FL. 33518.

TABLE 1. DIET COMPOSITION[a]

Item	%
Oats (IFN 4-03-309)	95.3
Molasses (IFN 4-04-696)	3.0
Limestone (IFN 6-02-632)	1.0
Trace mineral salt[b]	.5
Vit A, D, E and Se Premix[c]	.2

[a]As fed basis.

[b]Composition: NaCl (98.5 %), Fe (.232 %), Mn (.225 %), Zn (.200 %), Mg (.100 %), S (.040 %), Cu (.023 %), Co (.007 %) and I (.007 %).

[c]Supplied 5640 IU vit A (Control lambs only), 423 IU vit D, 100 IU vit E and .2 ppm Se per day.

Blood was obtained via jugular puncture immediately prior to and 1, 2 and 3 wk after immunization. Upon completion of the primary ovalbumin challenge period the lambs were given a secondary challenge of ovalbumin (1 mg) and a primary challenge of lysozyme (.1 mg). Both injections consisted of a one to one mixture of Freund's incomplete adjuvant and physiologic saline and were administered as before. Blood was again taken via jugular puncture prior to and 1, 2, and 3 wk post-challenge. At the end of this 3-wk period the lambs were given a secondary challenge of lysozyme (.1 mg) as before and blood was again taken prior to and 1, 2 and 3 wk post-challenge. Upon completion of the ovalbumin and lysozyme challenge periods, five blocks (10 animals) were chosen at random and sacrificed. At this time liver and spleen wt were obtained and liver samples taken and frozen (-20 °C) for later analysis of vitamin A.

The remaining 10 A-Def lambs were repleted with vitamin A and received the Con diet for the remainder of the experiment. Repletion consisted of 1 wk on the Con diet followed by a 100,000 IU oral dose of vitamin A palmitate. The lambs were then allowed an additional week on the Con diet. Following the 2-wk repletion period all lambs were challenged with .1 mg HGG in a mixture of .5 ml Freund's incomplete adjuvant and .5 ml physiologic saline. Half of the solution was again administered i.m. in the hind leg

with the remainder injected s.c. along the lateral thorax. Blood was taken prior to and 1, 2 and 3 wk post-challenge. A secondary challenge of HGG (.1 mg) was administered as before and blood drawn prior to and 1, 2 and 3 wk post-challenge. Upon completion of the HGG challenge periods, the animals were sacrificed and liver and spleen wt obtained. Liver samples were also obtained and frozen (-20 °C) for later analysis of vitamin A.

Trial II. Thirty crossbred wether lambs weighing approximately 18 kg were alloted by wt into 10 blocks. Two lambs per block were randomly assigned to the A-def diet and the remaining lamb assigned to the Con diet. Lambs were treated for parasites and vaccinatec against clostridia as in Trial I and housed similarly. The lambs were individually fed 900 $g \cdot d^{-1}$ of a whole oat diet (Table 1). A series of antigenic challenges was initiated when the mean serum vitamin A concentration of the A-def lambs fell below 20 $ug \cdot dl^{-1}$. The lambs were challenged with 20 ug of ovalbumin in a mixture of .5 ml Freund's incomplete adjuvant and .5 ml physiologic saline. This solution was injected as in Trial I and blood was obtained via jugular puncture immediately prior to the challenge and then weekly for 3 wk. A second challenge of ovalbumin (20 ug) was then administered as before except the antigen was suspended in 1 ml of physiologic saline. Freund's incomplete adjuvant

was not used in the secondary challenge. Blood was collected every other day for a week and then weekly for an additional 2 wk. Following the completion of the secondary ovalbumin challenge, one of the two A-def lambs in each block was chosen at random and repleted with vitamin A as in Trial I and remained on the Con diet until the termination of the study. Following the 2-wk repletion period, a second antigenic challenge was initiated. Twenty micrograms of HGG in .5 ml Freund's incomplete adjuvant and .5 ml physiologic saline was injected into the Con, A-def and A-repleted (A-rep) lambs as before. Blood was again collected prior to the challenge and weekly for 3 wk. A secondary challenge consisting of 20 ug HGG in 1 ml of physiologic saline was then injected as before. Blood was collected every other day for a week and then weekly for two subsequent weeks. The lambs were then sacrificed and liver and spleen wt were obtained. Liver samples were also taken and frozen (-20 °C) for latter analysis of vitamin A.

Throughout the challenge periods in both trials, serum was harvested and frozen for later analysis of vitamin A, cortisol, antigen-specific and polyclonal IgG levels. Heparinized blood samples were also drawn for the determination of packed cell volume (PCV).

Assay Procedures. Vitamin A was extracted by the method of Kimble (1939) and the color developed by the

trifluoroacetic acid method of Dugan et al. (1964). Liver vitamin A levels were determined as described by Gallup and Hoefer (1946). Serum cortisol concentrations were determined using a single antibody radioimmunoassay.[8]

Both antigen-specific and polyclonal IgG were determined using a modification of the enzyme-linked immunosorbant assay (ELISA) as described by O'Sullivan (1979). The concentration of antigen used to coat the microtiter plates was 5 $ug \cdot ml^{-1}$ for all antigen-specific IgG determinations. Initial serum dilutions for the ovalbumin and lysozyme challenges in Trial I were 1:50. Initial serum dilutions for the ovalbumin challenge in Trial II and the HGG challenges in both Trials I and II were 1:4. The enzyme labelled antibody used was a 1:5000 dilution of a peroxidase conjugated rabbit anti-sheep IgG (Fc)[9]. A .1 mmol solution of 2,2-Azainobis(3-ethylbenz-thiazoline sulfonic acid) (ABTS) was used as the substrate for the peroxidase enzyme. The reaction was allowed to proceed for 30 min at which time 5% sodium dodecyl sulfate (SDS) was added to each well to stop the reaction. The optical density of each well was determined with an microtiter plate reader[10] at 415 nm. A titer was realized

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[8]Amerlex Cortisol RIA Kit, Amersham Corp., Arlington Heights, Il. 60005.

[9]Pel-Freez Biologicals, Rogers , Ar. 72756.

[10]Titertek Multiscan MCC/340, Flow Laboritories, McClean Va. 22102

when the optical density of a given dilution was equal to that of the negative control.

For the determination of polyclonal IgG, microtiter plates were coated with a 5 ug·ml^{-1} solution of rabbit anti-sheep IgG (Fc)[11]. Serum samples were diluted 1:100,000 and run in triplicate. Standards containing known quantities of sheep IgG were also diluted 1:100,000 and a standard curve was run on each plate. Horseradish peroxidase conjugated rabbit anti-sheep IgG (Fc)[11] was used as the labelled antibody at a dilution of 1:5000. A .1 mmol solution of ABTS was again used as the substrate and the reaction was allowed to proceed for 30 min. After the reactions were stopped with SDS, optical densities were measured on an microtiter plate reader at 415nm.

Statistical Analysis. Treatment differences were analyzed by least-square analysis of variance utilizing the General Linear Model procedures of the Stastistical Analysis System (SAS, 1979). Treatment differences during the HGG challenges in Trial II were separated by orthoganol contrasts.

[11]Pel-Freez Biologicals, Rogers Ar. 72756.

Results and Discussion

In both trials the antigenic challenge periods did not begin until the mean serum vitamin A concentrations of the A-def lambs fell below 20 $ug \cdot dl^{-1}$. Serum vitamin A concentrations are not a precise indicator of vitamin A status, however, serum values below 20 $ug \cdot dl^{-1}$ are ackowledged as an indicator of vitamin A deficiency (May et al., 1987). Serum vitamin A concentrations prior to the primary ovalbumin challenge were greater ($P < .001$) in the Con lambs for both trials (Table 2). Serum vitamin A concentrations in the A-def lambs continued to decrease during the ovalbumin (Trial I and II) and lysozyme (Trial I) challenge periods while serum vitamin A concentrations for the Con lambs remained relatively constant. Following the 2-wk repletion period serum vitamin A concentrations for the A-rep lambs in both trials were similar to Con values and they remained the same to the end of both experiments. In Trial II serum vitamin A concentrations for the A-def lambs continued to decrease during the HGG challenge period.

Liver vitamin A concentrations in lambs sacrificed just prior to the repletion period in Trial I were 178.9 and 2.4 (+/- 10.2) $ug \cdot g^{-1}$ liver in the Con and A-def lambs,

Table 2. SERUM VITAMIN A CONCENTRATION

Treatment	Beginning of experiment[a] Mean	Prior to repletion[a] Mean	Following repletion[a] Mean	End of experiment[a] Mean
	ug·dl^{-1}			
Trial I				
Control	52.0[c]	58.3[c]	51.0	49.1
A-deficient	16.0	5.4		
A-repleted			48.3	48.9
SE[b]	8.2	5.9	7.3	11.4
Trial II				
Control	36.2[c]	45.1[c]	46.0[c]	44.6[c]
A-deficient	9.7	6.8	6.2	4.9
A-repleted			47.3[c]	41.9[c]
SE[b]	7.6	8.9	7.0	8.5

[a]Beginning of experiment = wk 1 (Trials I and II)
Prior to repletion = wk 10 (Trial I) and wk 7 (Trial II)
Following repletion = wk 12 (Trial I) and wk 9 (Trial II)
End of experiment = wk 18 (Trial I) and wk 15 (Trial II)
[b]Standard error of the mean.
[c]Con and/or A-rep lambs differ from A-def within each trial ($P < .001$).

respectively. At the completion of Trial I liver vitamin A concentrations were 178.8 $ug \cdot g^{-1}$ liver for the Con lambs and 130.0 (+/- 11.5) $ug \cdot g^{-1}$ liver for the A-rep lambs. Liver vitamin A concentrations in Trial II were 92.3 and 53.0 (+/- 8.4) $ug \cdot g^{-1}$ liver for the Con and A-rep lambs, respectively. The A-def lambs in Trial II had liver vitamin A concentrations of 1.7 (+/- 8.4) $ug \cdot g^{-1}$.

In both trials the A-def lambs had serum vitamin A concentrations indicative of a vitamin A-deficiency which was supported by very low hepatic vitamin A levels. A 2-wk repletion period was enough to elevate the A-def lambs' serum vitamin A concentrations to control values. Liver vitamin A concentrations for the A-rep lambs in both trials were lower ($P < .01$) than Con values even after 7 wk on the Con diet but were within a normal level.

Weights of the Con, A-def and A-rep lambs were almost identical throughout the two trials (Table 3). Studies involving vitamin A-deficient animals are sometimes confounded with protein-calorie malnutrition. This confounding makes interpreting the results difficult. The lambs in these two trials showed no growth depression or loss of appetite as a result of the vitamin A deficiency.

In Trial I IgG concentrations specific for ovalbumin increased in response to the ovalbumin challenge in both groups of lambs (Figure 1). The Con lambs had a numerically

Table 3. BODY WEIGHTS

Treatment	Beginning of experiment[a] Mean	Prior to repletion[a] Mean	Following repletion[a] Mean	End of experiment[a] Mean
Trial I	----------	----------	kg ----------	----------
Control	54.4	57.8	58.3	60.9
A-deficient	55.4	58.3		
A-repleted			58.1	59.5
SE[b]	.4	.4	.8	.9
Trial II				
Control	36.4	42.6	43.2	45.2
A-deficient	37.3	43.2	43.2	43.8
A-repleted			43.2	45.7
SE[b]	.6	.5	.4	.8

[a]Beginning of experiment = wk 1 (Trials I and II)
Prior to repletion = wk 10 (Trial I) and wk 7 (Trial II)
Following repletion = wk 12 (Trial I) and wk 9 (Trial II)
End of experiment = wk 18 (Trial I) and wk 15 (Trial II)
[b]Standard error of the mean.

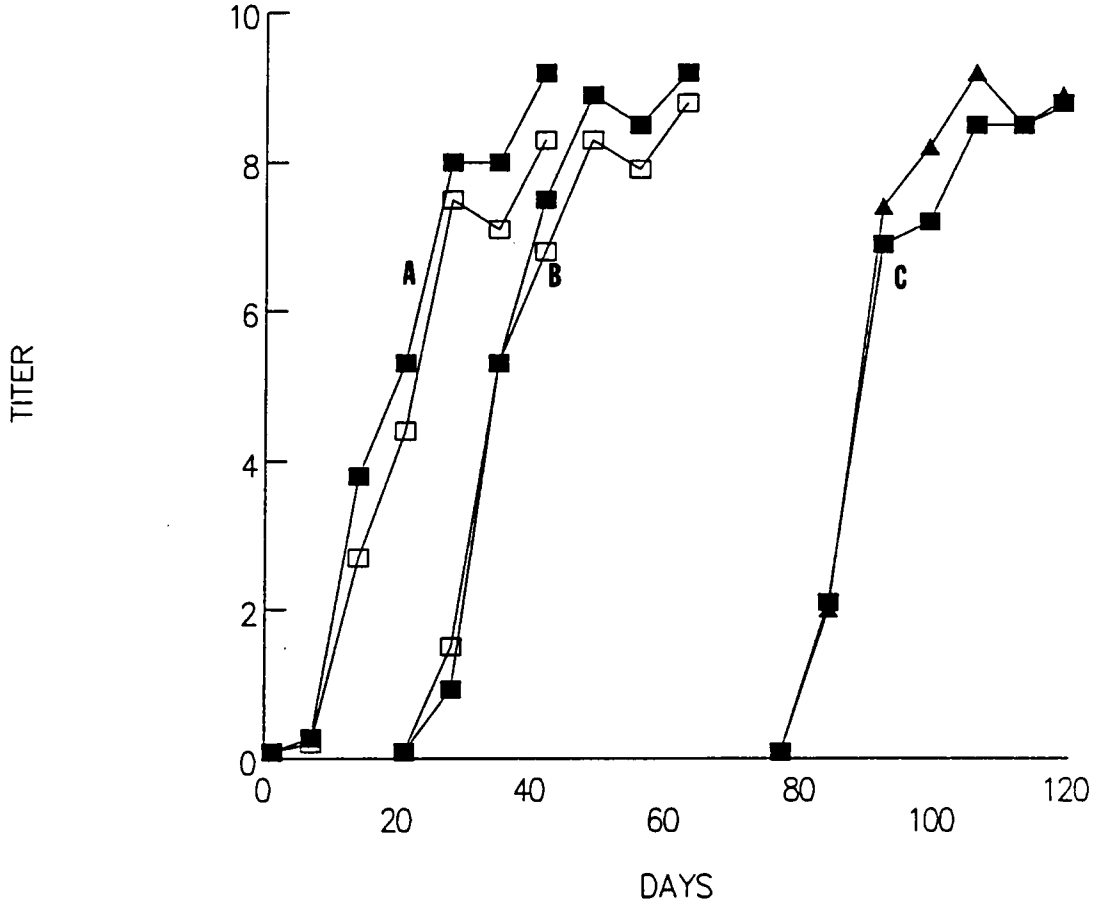

Figure 1. Serum IgG titers (log_2) specific for ovalbumin (**A**), lysozyme (**B**) and human gamma globulin (**C**) in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I). Initial serum dilutions for the ovalbumin and lysozyme challenges were 1:50. Initial serum dilution for the human gamma globulin challenge was 1:4.

greater serum concentration from d 14 to d 42. Titers were greater ($P<.09$) on d 42. Immunoglobulin G concentrations in response to the lysozyme challenge were similar to those of the ovalbumin challenge even with an injection concentration 1/10 that of the ovalbumin challenge. Titers were numerically greater in the control lambs through the second half of the challenge period (d 42 to d 63)

Following repletion of the A-def lambs in Trial I titers in response to a challenge of 100 ug of HGG were lower than either the ovalbumin or lysozyme challenges. Starting dilutions in the ELISA procedure for the determination of antigen-specific IgG were 1/50 for the ovalbumin and lysozyme challenges and 1/4 for the HGG challenge. Human gamma globulin appears to be less immunogenic to sheep than either ovalbumin or lysozyme and thus elicited a low humoral response. Both the Con and A-rep lambs in Trial I responded similarly with elevated serum IgG concentrations. There were no significant differences between treatments.

It has been suggested that antigen concentrations in amounts capable of eliciting large antibody responses may also mask dietary treatment effect (Herlyn and Glaser, 1976). Dietary treatment differences in antibody production are usually more pronounced in response to antigens given in low doses. This observation may in part explain the

varying results obtained when investigating the effects of vitamin A on antibody responses to various antigens.

In Trial II antigen dosages and routes of administration were modified in an attempt to elicit a less severe immune response. Starting dilutions for the determination of antigen-specific IgG in Trial II were 1/4 for both the ovalbumin and HGG challenge periods. Ovalbumin specific IgG titers in response to the primary injection of ovalbumin increased in both groups of lambs (Figure 2). By d 21 the titers of the control lambs appeared to plateau whereas the A-def lambs IgG titers continued to increase. The A-def lambs had a greater concentration ($P < .07$) of ovalbumin specific serum IgG by d 21. The difference in titers between the control and A-def lambs on d 21 may be the result of varying antigen clearence rates. If the control lambs were more efficient in clearing the ovalbumin there would be less antigen present to stimulate antibody production and in turn antibody concentrations would decline more quickly. If the A-def lambs, conversely, had a less efficient clearence rate the antigen would be present longer to stimulate antibody production and titers would decline less quickly. It has been observed that mice supplemented with vitamin A have a greater blood clearance rate of bacteria than normal mice (Cohen and Elin, 1974). Ovalbumin-specific IgG titers were greater in the control

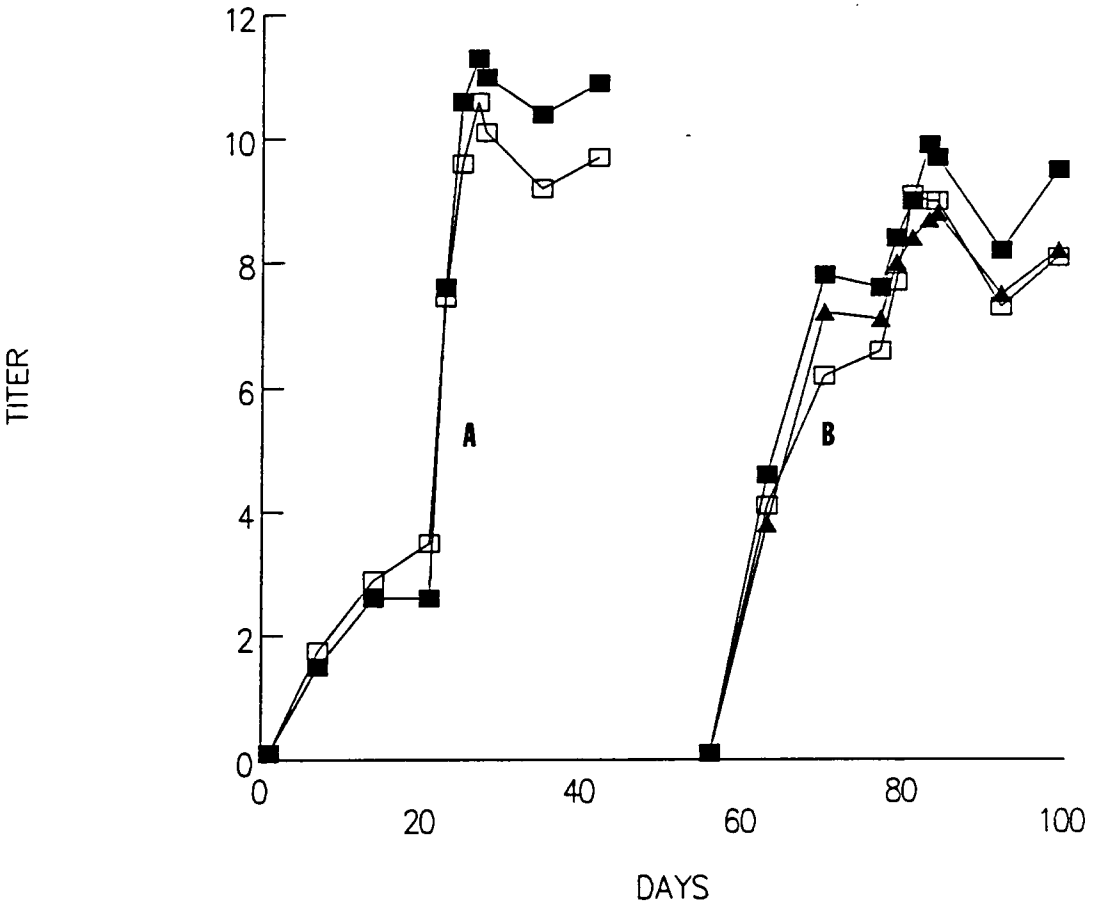

Figure 2. Serum IgG titers (log_2) specific for ovalbumin (**A**) and human gamma globulin (**B**) in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II). Initial serum dilutions for both challenge periods were 1:4.

lambs following the second ovalbumin challenge. Titers for control lambs were greater on d 25 and d 28 ($P<.07$) and on d 35 and d 42 ($P<.03$).

In response to the primary challenge of HGG in Trial II antibody titers for the control, A-def and A-rep lambs were not significantly different. Treatment effects, however, were observed following the secondary challenge. The control lambs had greater IgG titers than either the A-def or A-rep lambs on d 83 ($P<.01$), 92 ($P<.10$) and d 99 ($P<.003$). Repletion of the A-def lambs seemed to have no effect on antigen-specific antibody production as serum vitamin A concentrations were not different between the Con and A-rep lambs. Vitamin A-deficient and A-rep lambs exhibited similar HGG specific titers throughout the primary and secondary challenge periods. This finding may suggest that some secondary factor associated with vitamin A-deficiency may be the cause of the depressed antibody titers and not vitamin A itself. It may also be that the effect associated with a vitamin A deficiency takes longer than 2 wk to correct. If a cell population for instance was modified as a result of the vitamin A deficiency, it may take time for that population to turnover before an effect due to the repletion is observed. In either respect the repletion protocol used in trial II was not sufficient to restore antibody responses to Con levels even though serum

vitamin A concentrations were similar. The higher HGG dosages administered in Trial I may have masked any treatment effects. Vitamin A-rep lambs in Trial I also received vitamin A supplementation above that received in the ration. This added supplementation may have been necessary to restore antibody responses to Con levels.

In both trials treatment differences seemed to be more apparent during the secondary challenge periods. These treatment differences may become more distinct at lower antigen concentrations. It is important to point out, however, that differences between trials, or dosages can not be fully elucidated from the effects due to sex.

Serum polyclonal IgG concentrations in Trial I were not different between treatments (Figure 3). Polyclonal antibody concentration fell at the beginning of the ovalbumin challenge period and then declined no further throughout the remainder of the experiment. Vitamin A status seemed to have no effect on polyclonal serum IgG concentrations in Trial I. Treatment differences were observed, however, in Trial II (Figure 4). Polyclonal serum IgG concentrations during the ovalbumin challenge period were not different between treatments but the A-def lambs had numerically higher concentrations throughout the challenge period. Polyclonal serum IgG concentrations during the HGG challenge periods were similar for the

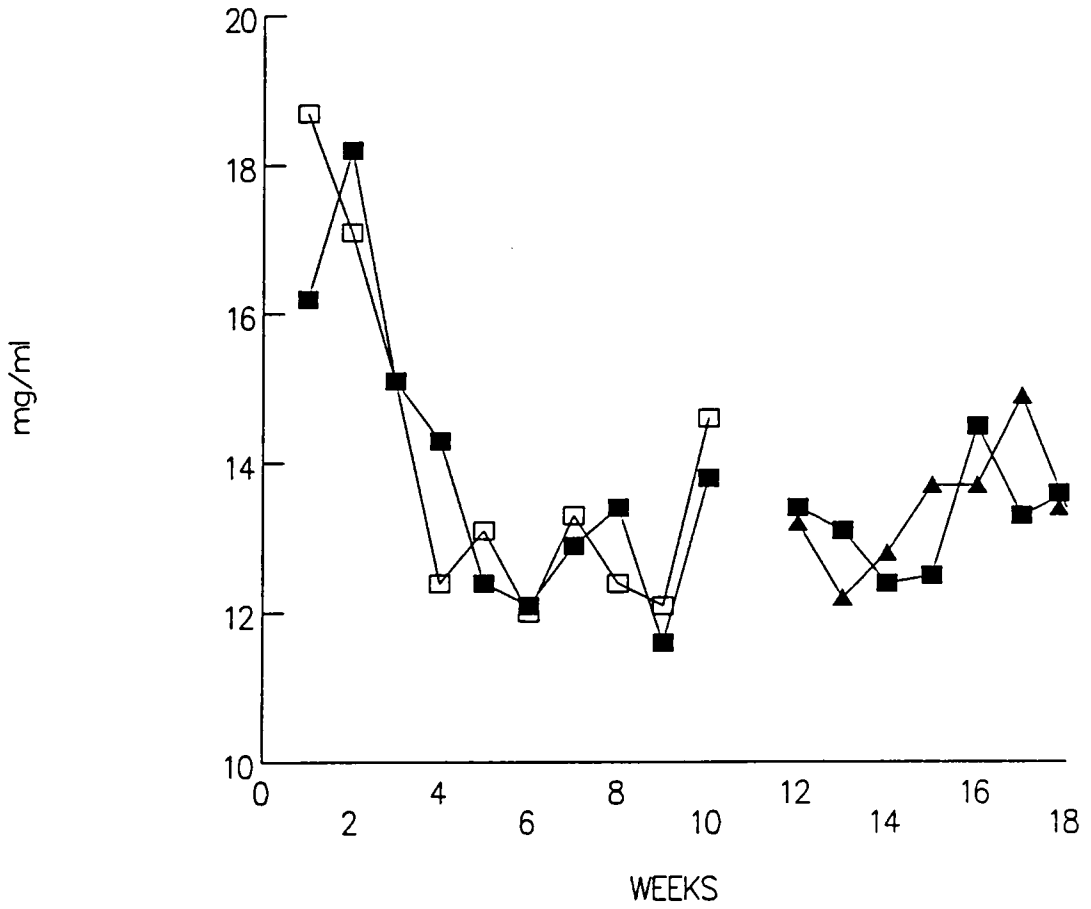

Figure 3. Serum polyclonal IgG concentrations in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I).

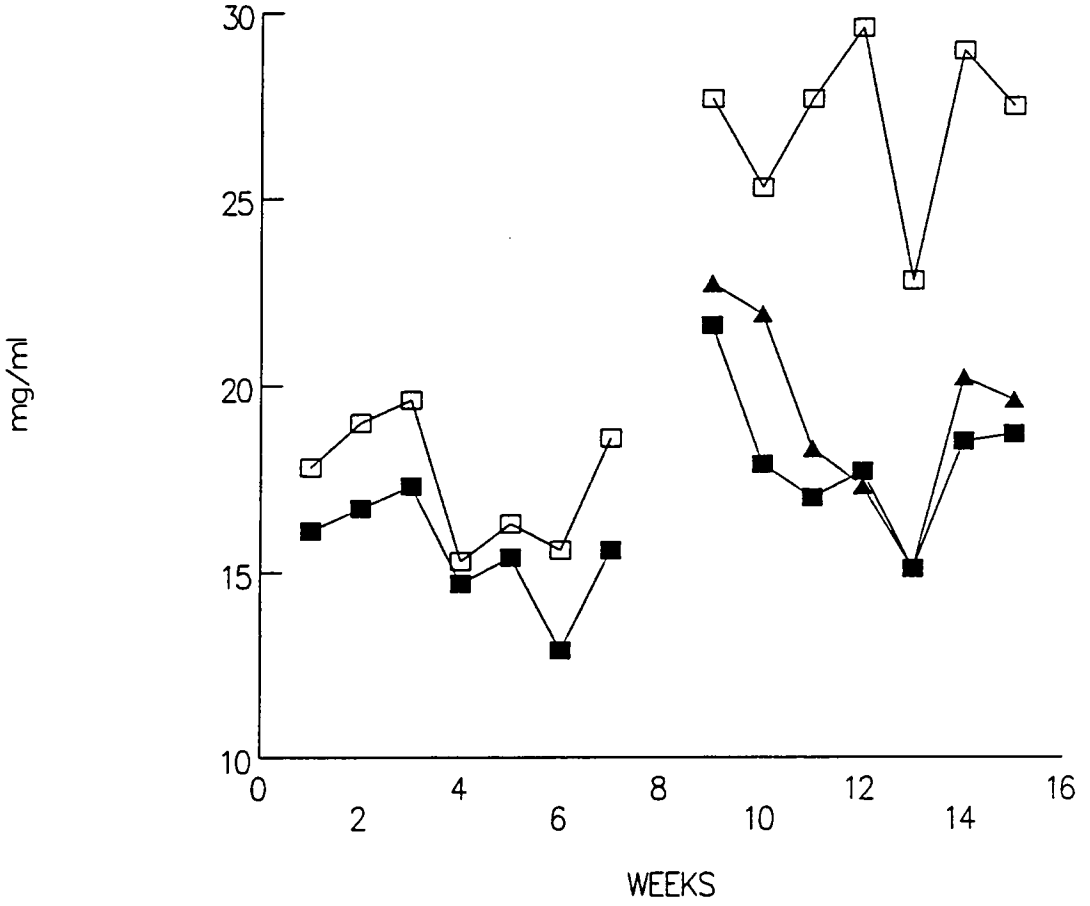

Figure 4. Serum polyclonal IgG concentrations in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II).

control and A-rep lambs. Serum polyclonal IgG concentrations of the A-def lambs rose markedly during this time. Concentrations were greater ($P < .05$) in the A-def lambs as compared to the control and A-rep lambs throughout the HGG challenge period in Trial II.

Elevated polyclonal serum IgG concentrations in vitamin A-deficient animals have been observed by others (Gershwin et al., 1984) and numerous theories have been proposed to explain this phenomenon. A shift in the T helper/suppressor ratio favoring the T helper cells has been proposed but so far evidence has remained inconclusive (Seshi and Purtilo, 1984). The increase in polyclonal serum IgG concentrations may be a function of an increased infection rate. Vitamin A deficiency is associated with a decrease in the integrity of epithelial linings. A breakdown in the first line of defense against bacterial and viral infections could result in an increase in infection rate. The greater the infection rate the greater the number of antigens present to cause polyclonal activation of B-cells and thus an elevated concentration of polyclonal serum IgG.

Packed cell volume in Trial I prior to the repletion period (through wk 10) was similar between treatments (Figure 5). After repletion of the A-def lambs, PCV for those lambs seemed to decrease. The PCV for the Con lambs remained relatively similar throughout the deficient and

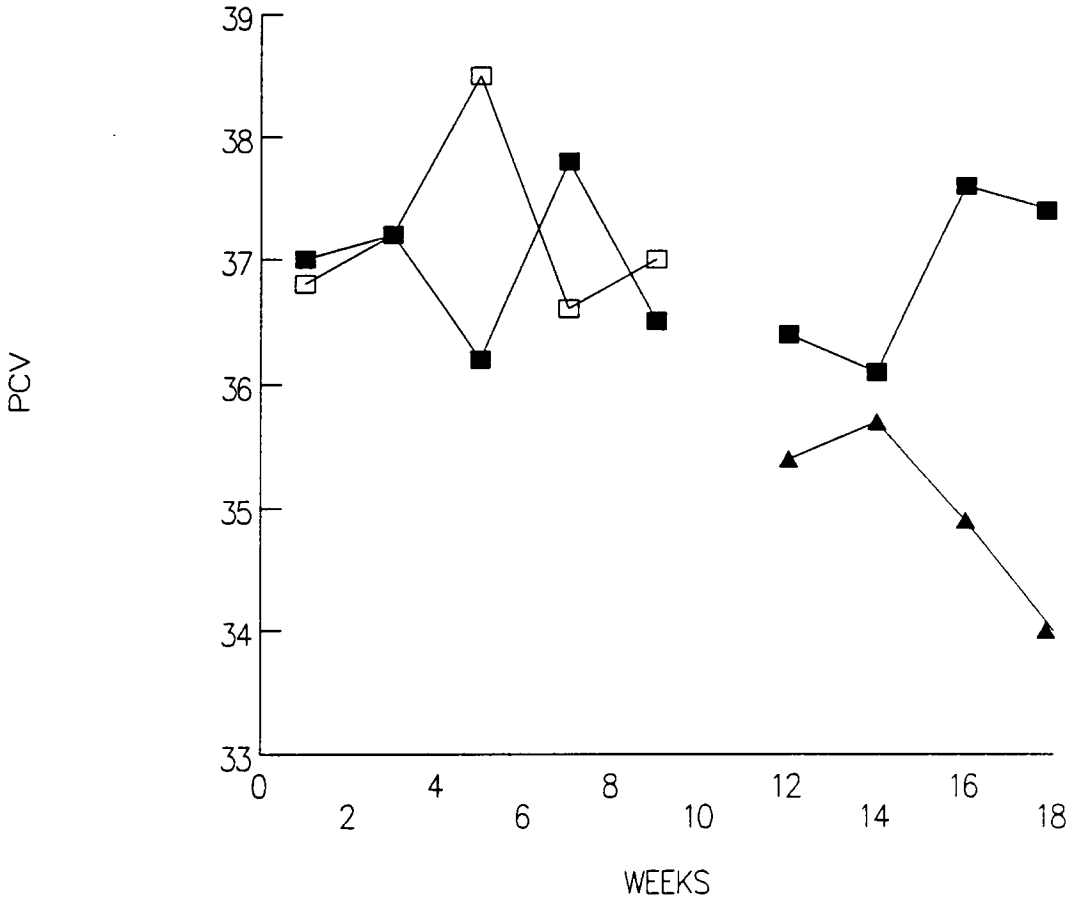

Figure 5. Packed cell volume in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I).

repletion periods. By wk 18 the A-rep lambs had a lower PCV ($P<.05$) than the Con lambs. Packed cell volume in Trial II varied from week to week but was unaffected by treatment (Figure 6). There was a tendency for A-def lambs to have higher PCV than either Con or A-rep lambs. The animals were bled every other day between wk 4 and wk 5 and this may have contributed to the drop in hematocrit on wk 5. The A-rep lambs in Trial II showed no decline in hematocrit as did the A-rep lambs in Trial I. It is possible that sex differences could have been responsible for the differences observed between the A-rep lambs in Trial I and II.

Spleen wt were unaffected by treatment in Trial I (Table 4). In Trial II the A-def lambs had a greater spleen wt ($P < .01$) than either the Con or A-rep lambs (Table 4). The increase in spleen wt in A-def lambs is consistent with observations made previously in this lab (Bruns, 1986). Nauss et al. (1979) however, observed decreased spleen wt in vitamin A-deficient rats. Increased spleen wt in vitamin A-deficient lambs may relate to increased polyclonal serum IgG also observed in the deficient lambs. If this relationship is real, increased numbers of plasma cells in the spleen would explain both effects. Further studies would be necessary to substantiate this possibility.

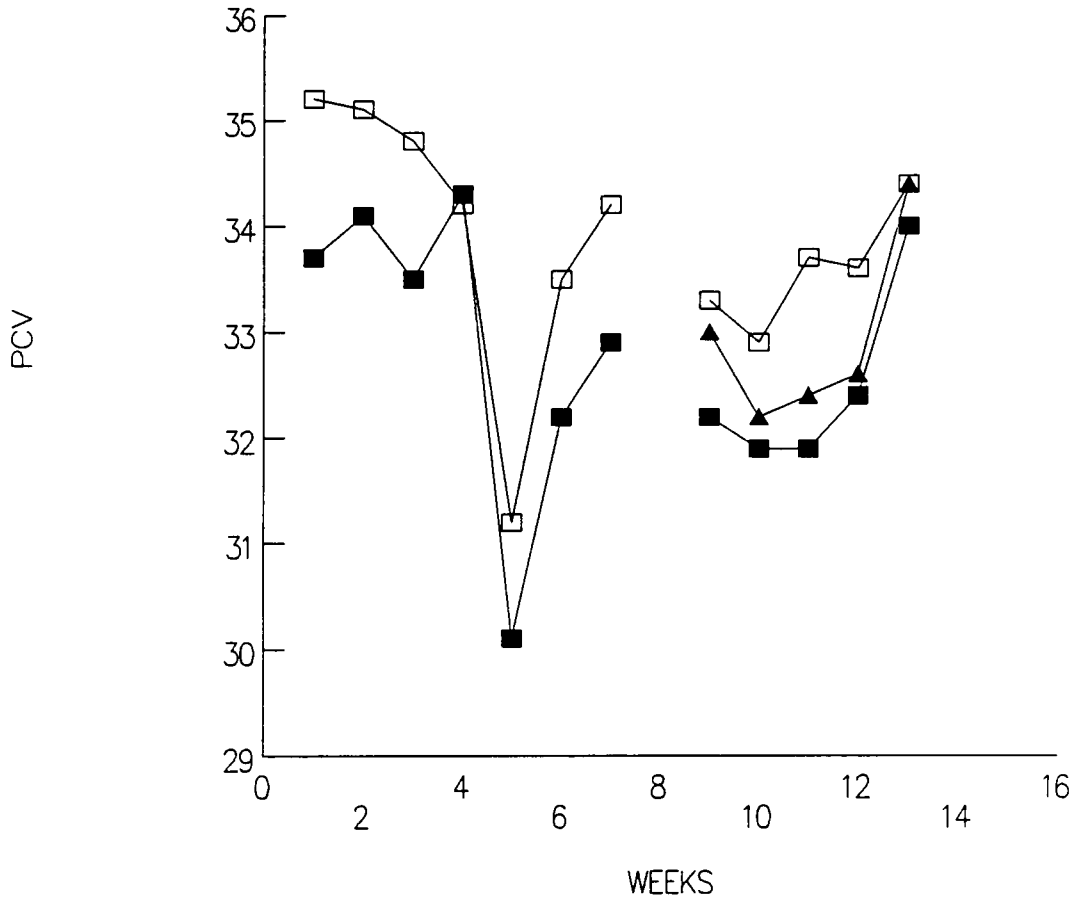

Figure 6. Packed cell volume in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II).

TABLE 4. EFFECTS OF VITAMIN A STATUS ON GROSS SPLEEN AND LIVER WEIGHTS

	Organ weights (% body wt)								
	Trial I						Trial II		
	Prior to repletion[a]			End of experiment[a]			End of experiment[a]		
Organ	Con	A-def	SE[b]	Con	A-rep	SE[b]	Con	A-def	A-rep	SE[b]
Spleen	.133	.140	.010	.130	.133	.007	.133	.159[d]	.135	.007
Liver	.973	.982	.026	.874	.811[c]	.025	.877	1.100[•]	.849	.037
n =	5	5		10	10		10	10	10	

[a]Prior to repletion = wk 10
End of experiment = wk 18 (Trial I) and wk 15 (Trial II)
[b]Standard error of the mean.
[c]A-repleted differ from Control ($P < .10$).
[d]A-deficient differ from Control and A-repleted ($P < .01$).
[•]A-deficient differ from Control and A-repleted ($P < .001$).

Gross liver wt in Trial I were greater ($P < .10$) in the Con lambs as compared to the A-rep lambs (Table 4.). In Trial II, A-def lambs had a greater liver wt ($P < .001$) than either the Con or A-rep lambs (Table 4) while Con and A-rep lambs had similar liver wt. The tendency for A-def lambs to have greater liver wt and A-rep lambs to have smaller liver wt is not observed in all vitamin A-deficient animals. Nauss et al., (1979) and Gershwin et al., (1984) both observed tendencies for A-def mice to have smaller liver wt. Species differences or differences in the severity of the deficiency may be two explanations for the conflicting results found from earlier experiments.

Results from this study indicate that antigen-specific IgG responses are reduced in vitamin A-deficient lambs and this reduction was more obvious at lower antigen dosages and on secondary antigenic challenges. A 2-wk repletion of A-def lambs as performed in trial II was not sufficient to restore humoral immune function equivalent to Con lambs. The reduction in humoral immune function in the A-rep lambs was observed even though serum vitamin A concentrations were not different from Con lambs and liver vitamin A levels were quite adequate. This suggest a lag phase between serum and liver vitamin A repletion and a return to normal immune function. One possible explanation is that the lymphocyte population may have been permanently

modified in the A-def lambs which were later repleted. Serum polyclonal IgG concentrations in A-def lambs during the HGG challenge periods in Trial II were elevated and gross spleen wt were also greater in these same A-def lambs. In summary, vitamin A-deficiency in lambs causes a reduction of humoral response to specific antigens particularly during the secondary exposure and a generalized increase in polyclonal IgG production.

Chapter IV

Cell-Mediated Immunity In Vitamin A-Deficient Lambs

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ABSTRACT

In vitro peripheral blood lymphocyte proliferation was measured in control (Con), vitamin A-deficient (A-def) and vitamin A-repleted (A-rep) lambs. Optimal and suboptimal concentrations of concanavalin A (ConA), phytohemaglutinin (PHA) and the antigens ovalbumin, lysozyme and human gamma globulin (HGG) were used to stimulate in vitro lymphocyte proliferation. Unstimulated lymphocyte proliferation in Trial I was greater ($P < .08$) in the A-def lambs prior to repletion and numerically greater in the A-rep lambs after repletion but not different between treatments in Trial II. Lymphocyte proliferation stimulated by an optimal concentration of ConA was not different between treatments in Trial I but was greater in the A-def lambs during the

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HGG challenge period in Trial II. Less than optimal concentrations of ConA elicited proliferations that were lower ($P < .07$) in the A-rep lambs in Trial I and higher in the A-def lambs ($P < .07$) (HGG challenge) in Trial II. Proliferations in response to optimal PHA concentrations were unaffected by treatment in Trial I but tended to be greater during the HGG challenge in the A-def lambs in Trial II. Suboptimal PHA stimulated lymphocyte proliferation was lower in the A-def lambs in Trial I ($P < .06$) and higher ($P < .09$) in the A-def lambs in Trial II (HGG challenge period). Antigen-stimulated lymphocyte proliferation was not different between treatments in Trial I. In Trial II ovalbumin stimulated lymphocyte proliferation was not different between treatments but the A-def lambs tended to have greater lymphocyte proliferation in response to HGG. Serum cortisol concentrations in the A-rep lambs tended to be lower than Con lambs in both trials. Stimulated lymphocyte proliferation was not significantly affected by vitamin A status in trial I. The lymphocytes from A-def lambs in Trial II, however, exhibited elevated proliferation levels during the second half of the experiment.

(Key words: Vitamin A, Cell-Mediated Immunity, Concanavalin A, Phytohemaglutinin)

Introduction

Cell-mediated immunity refers to that part of the immune system associated with thymic-derived lymphocytes (T-cells). T-cells are necessary for providing "help" to bone marrow-derived lymphocytes (B-cells) for the production of antibodies (Ab). T-cells are also involved in the lysis of virally infected target cells and in the suppression of the immune response. Researchers in the last two decades have observed an interaction between proper T-cell function and nutritional status in various animals. Vitamin A has been implicated to exert effects on proper T-cell function. Mitogenic responses of splenic lymphocytes in vitamin A-deficient rats were significantly less than pair fed controls. Vitamin A supplementation of A-deficient mice for 3 d restored the spleen cell mitogen responses back to normal (Nauss et al. 1979). Retinoids inhibit the growth and development of certain types of tumors (Patek et al. 1979). Low doses of retinoic acid increase the cytolytic activity of spleen cells against syngeneic tumor cells (Glaser and Lotan 1979). Vitamin A given to cancer patients increases the mitogenic response by lymphocytes (Micksche et al. 1977). Recent findings suggest that supplementation of 13-cis retinoic acid to humans can

significantly lower the plasma concentration of the immunosuppressive hormone, cortisol (Watson et al. 1986).

The objective of this study was to evaluate the in vitro peripheral blood lymphocyte proliferation to concanavalin A (ConA), phytohemaglutinin (PHA) and specific antigens in control, vitamin A-deficient and vitamin A-repleted lambs. Serum cortisol concentrations also were monitored throughout the study to determine if vitamin A status could affect this immunosuppressive hormone.

Experimental Procedure

Trial I. Thirty crossbred ewe lambs weighing approximately 30 kg were blocked according to wt and randomly assigned to either a control (Con) or vitamin A-deficient (A-def) treatment. The lambs were treated for internal parasites (levamisole[5]) and vaccinated against multiple clostridial species (Ultrabac-7[6]). The lambs were housed individually under constant lighting conditions in raised pens (.8 x 1.0 m) with expanded metal floors and equipped with automatic nipple waterers. The lambs were fed once daily at the rate of 900 $g \cdot head^{-1} \cdot d^{-1}$ of a whole oat diet (Chapter 4). In addition to that supplied in the diet,

[5]Pitman-Moore, Washington Crossing, NJ. 08560.
[6]Beecham Laboratories, Bristol, TN. 37620.

the control lambs received a 100,000 IU oral dose of vitamin A palmitate[7] in capsule form every 2 wk. When the mean serum vitamin A concentration of the A-def lambs fell below 20 $ug \cdot dl^{-1}$ the lambs were exposed to series of antigenic challenges as described previously (Chapter 4). Briefly, all lambs were given primary and secondary antigenic challenges of ovalbumin (1 mg) and lysozyme (.1 mg). The antigens were administered in a one to one mixture of Freund's incomplete adjuvant and physiologic saline. Ovalbumin was injected on wk 1 and 4 while lysozyme was injected on wk 4 and 7. Blood was obtained weekly via venapuncture during both challenge periods.

Upon completion of the ovalbumin and lysozyme challenge periods, ten blocks (20 animals) were chosen at random to continue in the trial. Of the animals continuing, 10 A-def lambs were repleted with vitamin A and received the Con diet for the remainder of the experiment. Repletion consisted of 1 wk on the Con diet followed by a 100,000 IU oral dose of vitamin A palmitate. The lambs were then allowed an additional week on the Con diet. Following the 2-wk repletion period all lambs were injected with primary and secondary antigenic challenges of HGG (.1 mg). The antigen was administered in a one to one mixture of Freund's incomplete adjuvant and physiologic saline. The

[7]R.P. Scherer Corp., Clearwater, FL. 33518.

lambs were injected on wk 12 and 15 and blood was obtained weekly via venapuncture.

Trial II. Thirty crossbred wether lambs weighing approximately 18 kg were alloted by wt into 10 blocks. Two lambs per block were randomly assigned to be depleted of vitamin A and the remaining lamb assigned to maintain a normal vitamin A status. Lambs were treated for parasites and vaccinated for clostridia and housed as in Trial I. The lambs were individually fed 900 $g \cdot d^{-1}$ of a whole oat diet (Chapter 4). A series of antigenic challenges was initiated when the mean serum vitamin A concentration of the A-def lambs fell below 20 $ug \cdot dl^{-1}$. The lambs were given primary (wk 1) and secondary (wk 4) antigenic challenges of ovalbumin (20 ug) as described previously (Chapter 4) and bled weekly via venapuncture. The primary challenge was administered in a one to one mixture of Freund's incomplete adjuvant and physiologic saline while the secondary challenge was administered in physiologic saline. Following the completion of the secondary ovalbumin challenge, one of the two A-def lambs in each block was chosen at random and repleted with vitamin A as in Trial I and remained on the Con diet until the termination of the study. Following the 2-wk repletion period a second antigenic challenge was initiated. Primary (wk 9) and secondary (wk 12) antigenic challenges of HGG (20 ug) were injected into Con, A-def and

A-repleted (A-rep) lambs. The primary challenge was administered in a one to one mixture of Freund's incomplete adjuvant and physiologic saline while the secondary challenge was administered in physiologic saline. Blood was collected weekly during both the primary and secondary challenge periods.

Throughout the challenge periods in both trials serum was harvested and frozen for later analysis of cortisol. Serum cortisol concentrations were determined using a single antibody radioimmunoassay[8].

Lymphocyte proliferation. Heparinized blood samples were obtained weekly for the separation of peripheral blood lymphocytes which were used to determine the in vitro lymphocyte proliferation (Bradley, 1980). Peripheral blood lymphocytes were suspended in RPMI 1640 medium and 1 X 10^5 (Trial I) or 2 X 10^5 (Trial II) viable cells were added to each microtiter plate well. The culture medium in Trial I contained 10% lamb serum and in Trial II 10% fetal calf serum. Final concentrations of mitogens used in triplicate wells were: ConA 16 and 1.6 $ug \cdot ml^{-1}$, PHA 1:10 (Trial I), 1:20 (Trial II) and 1:500 (Trials I and II), ovalbumin, lysozyme and human gamma globulin (HGG) 1 and .1 $mg \cdot ml^{-1}$. One set of triplicate wells received only RPMI 1640.

[8]Amerlex Cortisol RIA Kit. Amersham Corp., Arlington Heights IL, 60005.

Mitogen-stimulated cells were cultured for 72 h. Antigen-stimulated cells were cultured for 72 h in Trial I and 120 h in Trial II. The cells were then pulsed with 1 uCi of tritiated thymidine per well and allowed an additional 16 h incubation. The cells were then harvested using a cell harvester[9] and tritiated thymidine incorporation was determined using a beta counter.

Statistical Analysis. Treatment differences were analyzed by least-square analysis of variance utilizing the General Linear Model procedures of the Statistical Analysis System (SAS, 1979). Treatment defferences during the HGG challenges in Trial II were separated by orthoganol contrasts.

Results and Discussion

Baseline (unstimulated) lymphocyte proliferation in Trial I was greater ($P < .08$) for the A-def lambs prior to repletion (Figure 1). Following repletion of the A-def lambs, baseline proliferation levels remained numerically higher and the mean differences in proliferation levels between the Con and A-def lambs increased through the repletion period (wk 12-18). Baseline lymphocyte

[9]Whittaker M.A. Bioproducts, Inc. Walkersville, MD 21793.

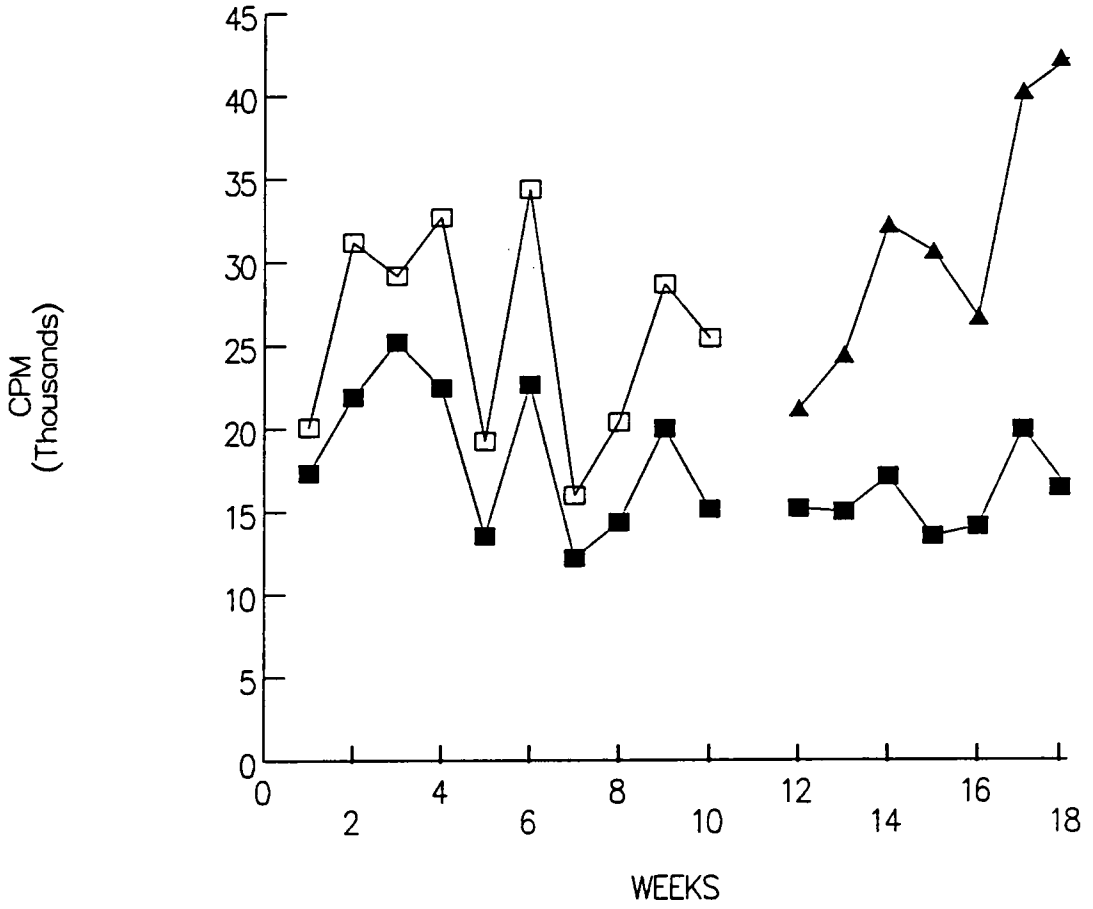

Figure 1. Unstimulated lymphocyte proliferation in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I).

proliferation levels in the Con lambs remained constant through the HGG challenge periods in Trial I while the lymphocyte proliferation levels of the A-rep lambs increased through the period resulting in a treatment by week interaction ($P < .007$). It is possible that an unidentified component(s) of the culture medium preferentially stimulated the lymphocytes from the A-def lambs while having little or no effect on Con lymphocytes. It is not known whether such a component(s), if it exists, is acting as a hormone or as an antigen. In Trial I the culture medium contained 10% autologous serum and in Trial II the culture medium contained 10% fetal calf serum. Baseline lymphocyte proliferation levels in Trial II were not different between treatments during either challenge period (Figure 2) except on wk 9 ($P < .14$), 10 ($P < .09$) and 11 ($P < .10$) when they were higher in the A-def group. The differences in proliferation levels between trials may be the result of the different sources of serum used in the culture medium. When autologous serum was used the A-def and A-rep lambs had greater baseline proliferation levels. When fetal calf serum was used in the culture medium there were no differences between treatments.

Because of the differences in baseline lymphocyte proliferation between treatments in Trial I, lymphocyte proliferation in response to mitogens and specific antigens

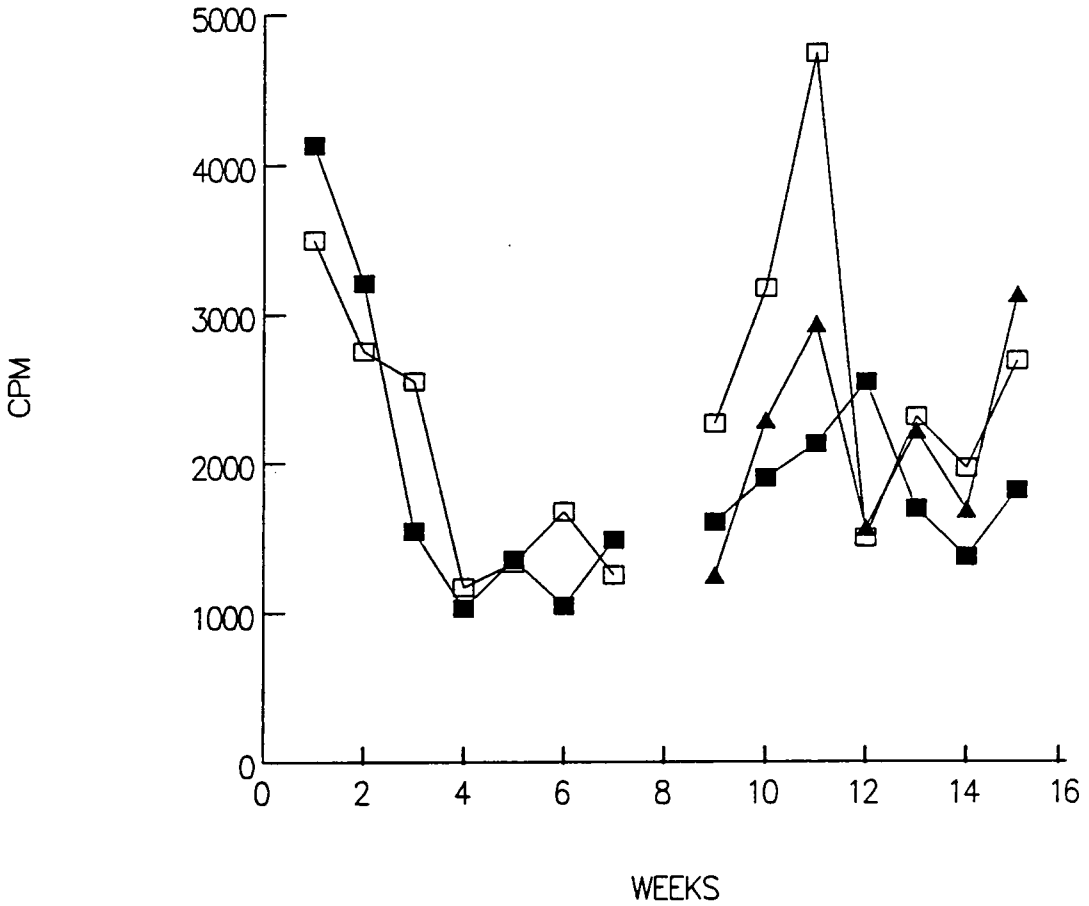

Figure 2. Unstimulated lymphocyte proliferation in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II).

will be described as corrected counts per minute (ccpm). Corrected counts per minute are the numerical differences between the cpm of the stimulated cells and the unstimulated cells. For consistency Trial II values will also be described in this manner.

Lymphocyte proliferation in response to 16 ug·ml^{-1} ConA fluctuated from week to week but was not different between treatments prior to repletion in Trial I (wk 1-10) or Trial II (wk 1-7) (Figures 3 and 4). Following vitamin A-repletion Con and A-rep lambs continued to show similar lymphocyte proliferation levels in response to optimal ConA concentrations. The A-def lambs during this same period (wk 9-15) in Trial II had ConA-stimulated lymphocyte proliferation levels greater ($P < .09$) than both the Con and A-rep lambs on all wk except 11 and 12.

Lymphocyte proliferation in response to a suboptimal concentration of ConA (1.6 ug·ml^{-1}) was not different between treatments prior to repletion in Trial I (Figure 5). Following repletion (wk 12-18) of the A-def lambs, however, proliferation was less ($P < .07$) than Con values. In Trial II lymphocyte proliferation in response to suboptimal concentrations of ConA was not different during the ovalbumin challenge period. During the HGG challenge periods (wk 9-15) the lymphocyte proliferation levels of the A-def lambs became greater ($P < .07$) than the Con or A-

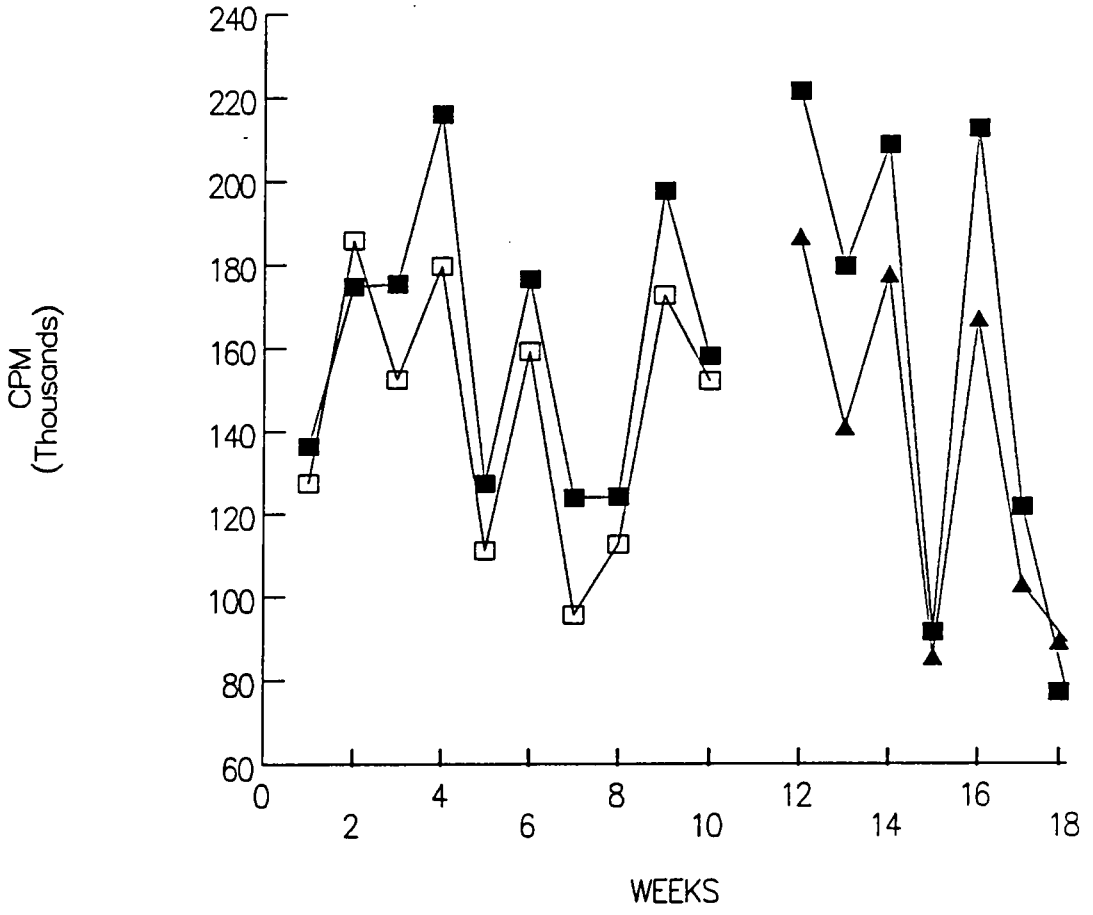

Figure 3. Lymphocyte proliferation in response to 16 ug·ml^{-1} concanavalin A in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I). Values above were corrected by subtracting baseline proliferation levels.

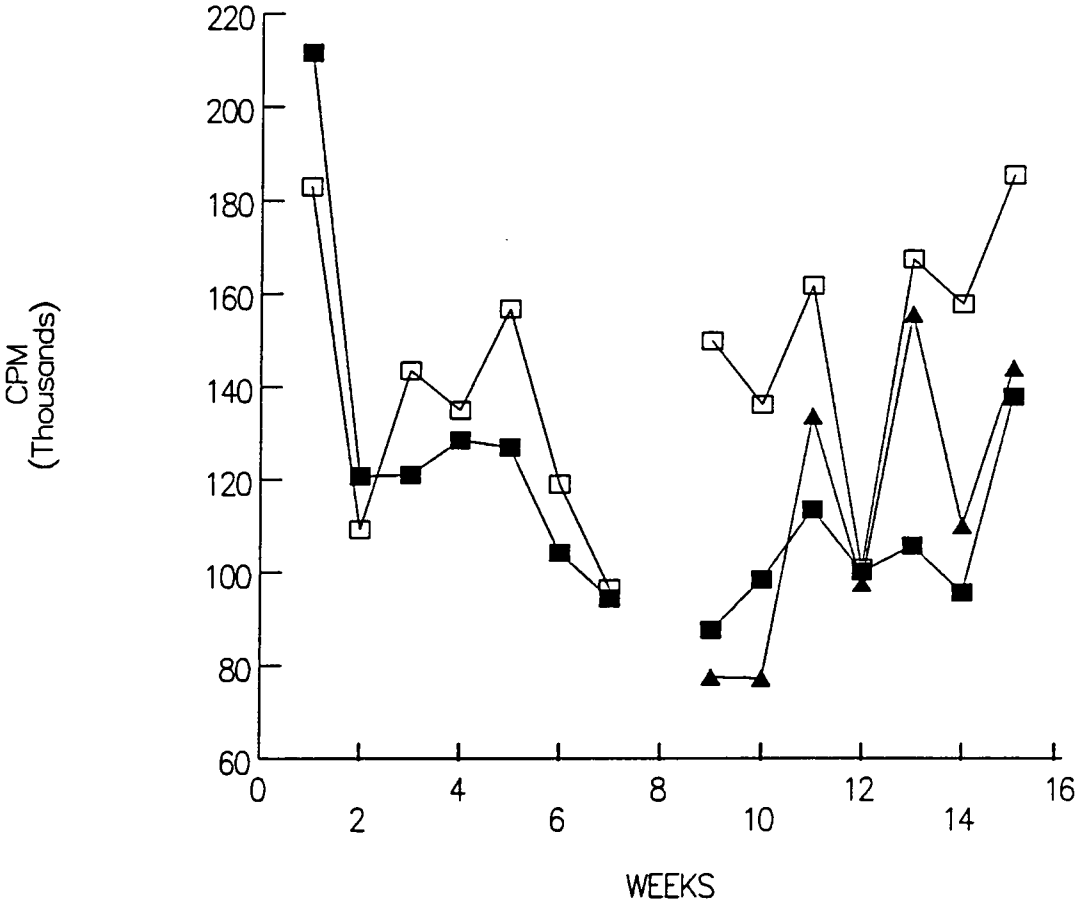

Figure 4. Lymphocyte proliferation in response to 16 ug·ml^{-1} concanavalin A in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II). Values above were corrected by subtracting baseline proliferation levels.

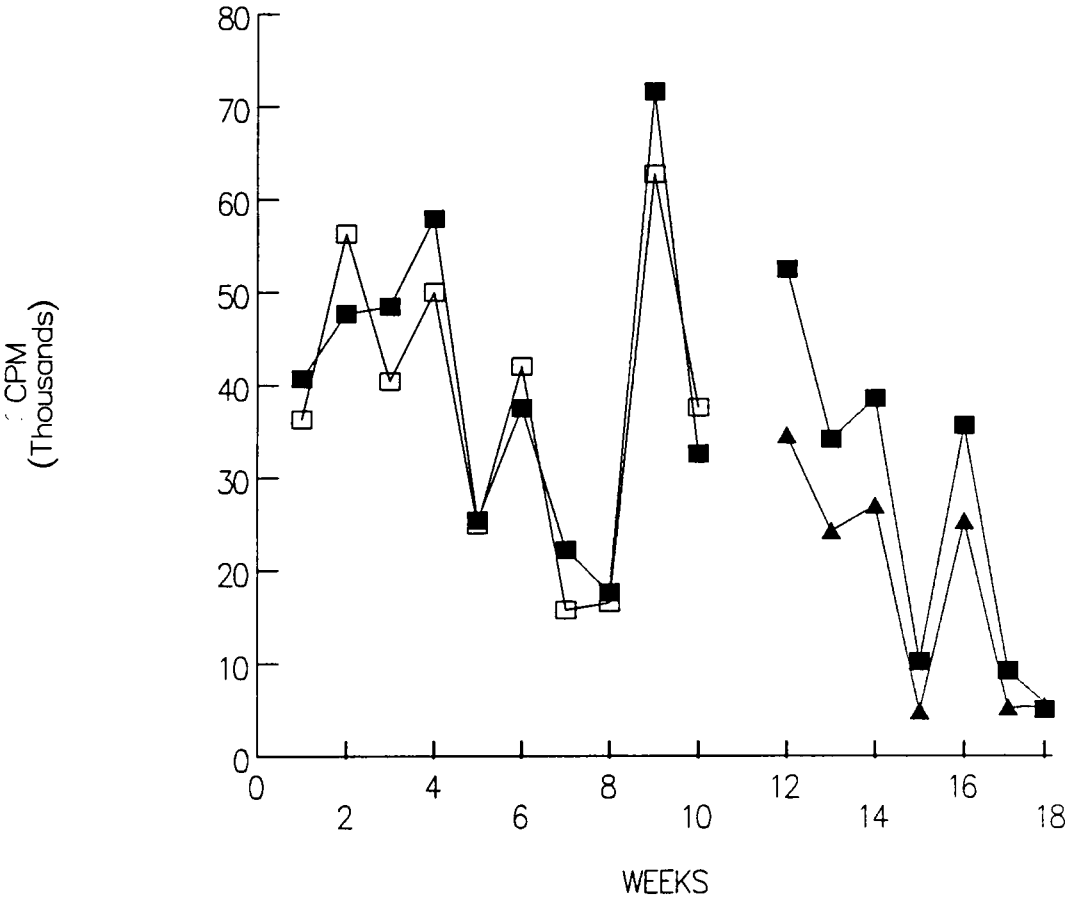

Figure 5. Lymphocyte proliferation in response to 1.6 ug·ml^{-1} concanavalin A in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I). Values above were corrected by subtracting baseline proliferation levels.

rep lambs (Figure 6). Control and A-rep lambs exhibited similar ConA-stimulated lymphocyte proliferation during the HGG challenge periods (wk 9-15) in Trial II. The differences observed in lymphocyte proliferation in response to suboptimal ConA concentrations between the Con and A-rep lambs in Trial I may be as much a consequence of mathematics as actual values. Because all stimulated proliferations were corrected for baseline proliferation levels, a very large baseline level may result in corrected cpm for the mitogen-stimulated lymphocytes that are lower than expected. The baseline proliferation levels for the A-rep lambs during the HGG challenge periods (wk 9-15) in Trial I were quite large and thus subtracting this value from the ConA-stimulated lymphocyte proliferations may result in apparent differences observed between treatments. The observation that lambs in Trial II had greater ConA-stimulated lymphocyte proliferation is contrary to observations made with vitamin A-deficient mice (Nauss et al., 1979). This discrepancy may be the result of species differences, differences in the severity of the deficiency or differences in the source of lymphocytes used in the assay. The source of lymphocytes used to measure proliferation may have an effect on the results. It has been observed that vitamin A-deficiency in mice resulted in a decrease in splenic lymphocyte proliferation while lymph

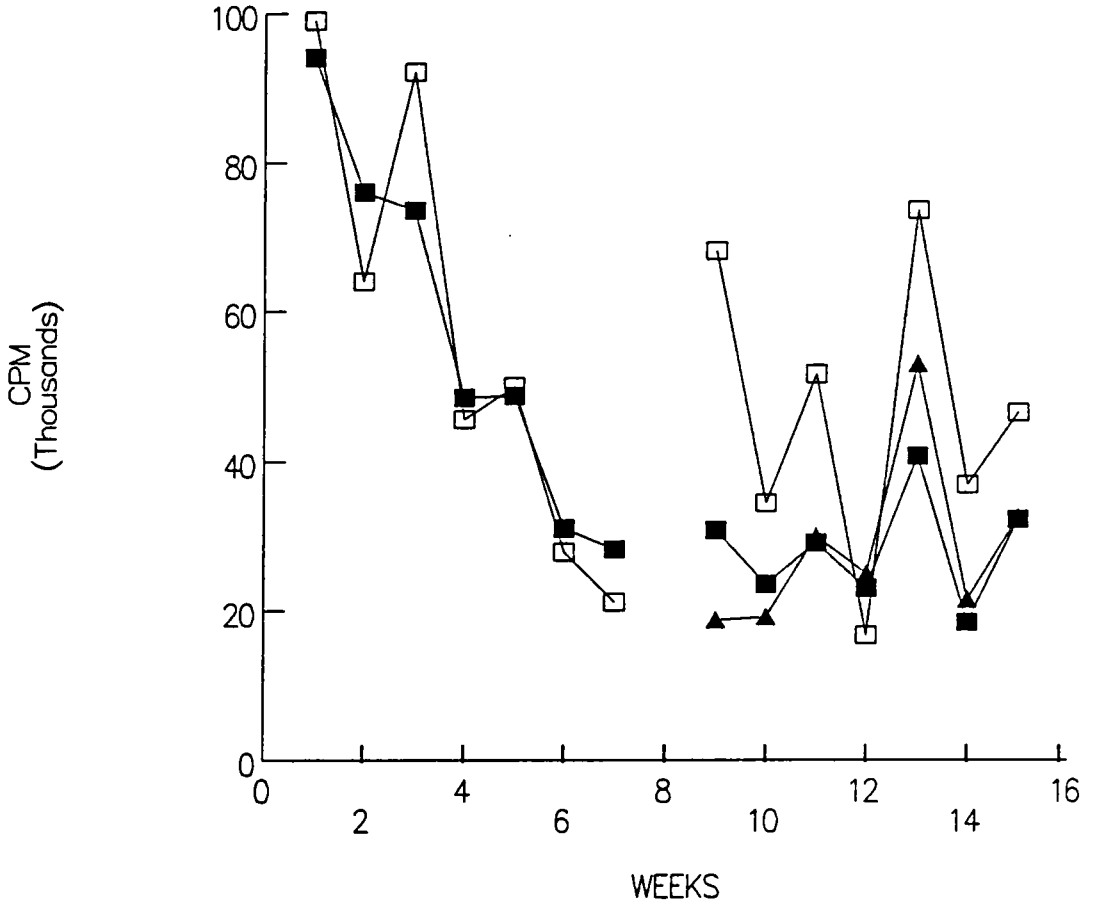

Figure 6. Lymphocyte proliferation in response to 1.6 ug·ml^{-1} concanavalin A in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II). Values above were corrected by subtracting baseline proliferation levels.

node lymphocytes from these same animals responded with normal or even elevated ConA-stimulated proliferations (Nauss et al., 1985). Peripheral blood lymphocytes in vitamin A-deficient chickens have greater ConA-stimulated lymphocyte proliferation than controls (Davis and Sell, 1983).

There were no treatment differences in the proliferation levels of lymphocytes stimulated with optimal concentrations (1:10) of PHA in Trial I (Figure 7). Proliferation levels fluctuated weekly and tended to rise as the experiment progressed. Optimal PHA (1:20) stimulated lymphocyte proliferation in Trial II was unaffected by treatment prior to repletion (wk 1-7) (Figure 8). There was a tendency, however, for the A-def lambs to have higher proliferation levels. During the HGG challenge period (wk 9-15) A-def lambs had a higher ($P < .10$) proliferation than either the Con or A-def lambs on all weeks except 12 and 13.

A suboptimal concentration of PHA (1:500) elicited a proliferation level that was lower ($P < .06$) in the A-def lambs prior to repletion in Trial I (wk 1-10) (Figure 9). Following repletion of the A-def lambs suboptimal PHA-stimulated proliferation tended to be lower but not significantly different from Con values. Proliferation levels in response to a suboptimal concentration of PHA in

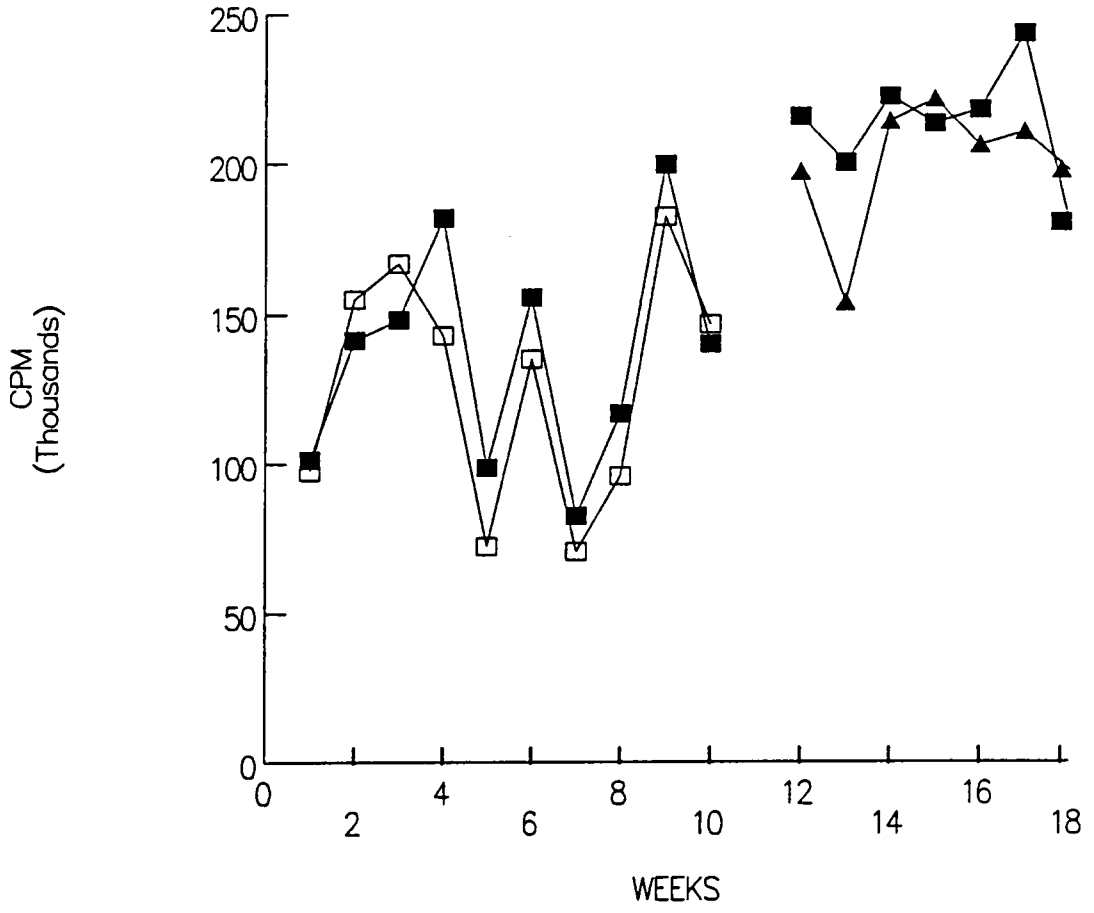

Figure 7. Lymphocyte proliferation in response to a 1:10 dilution of phytohemaglutinin in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I). Values above were corrected by subtracting baseline proliferation levels.

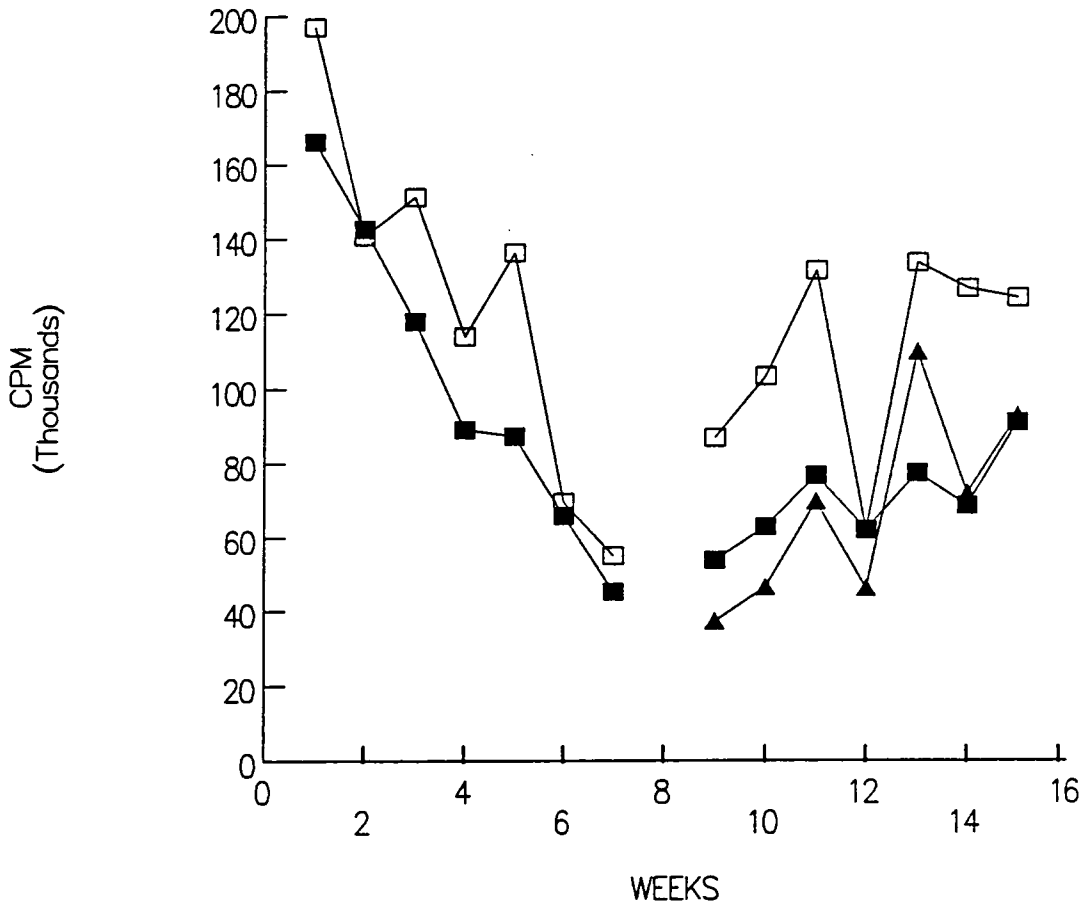

Figure 8. Lymphocyte proliferation in response to a 1:20 dilution of phytohemaglutinin in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II). Values above were corrected by subtracting baseline proliferation levels.

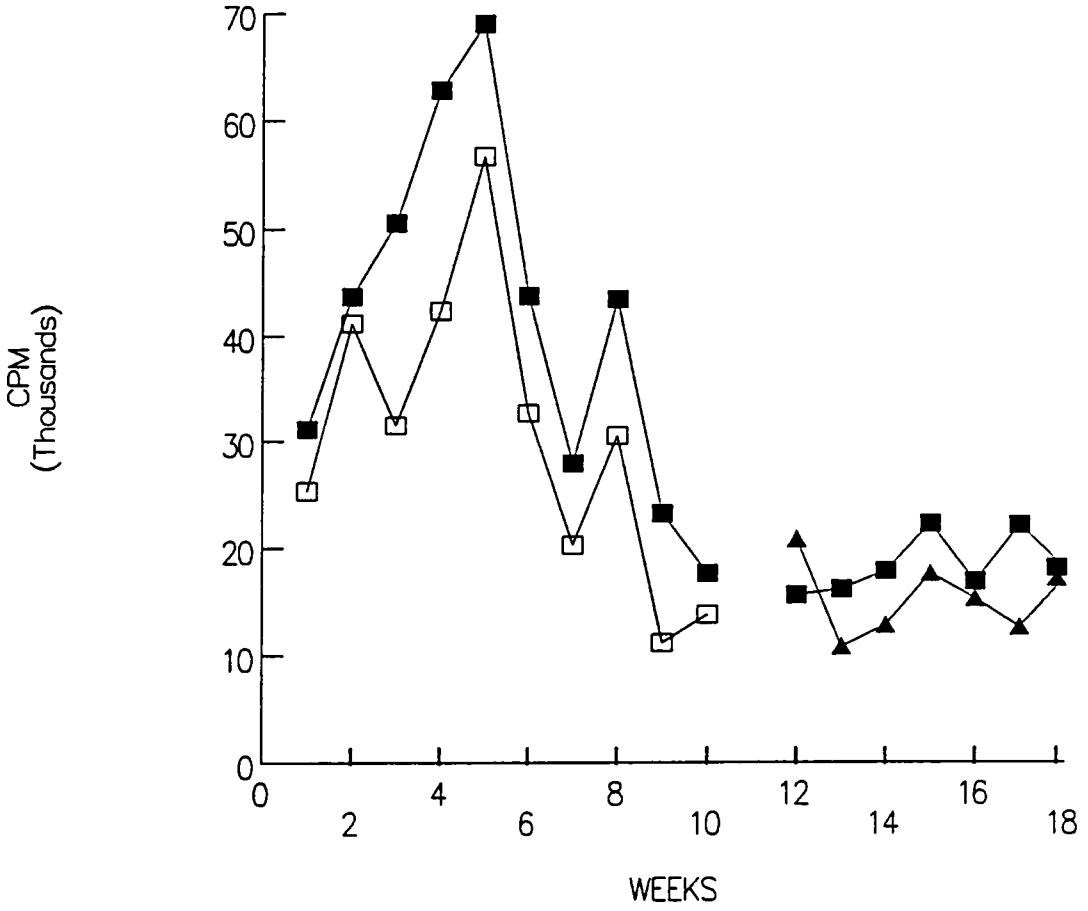

Figure 9. Lymphocyte proliferation in response to a 1:500 dilution of phytohemaglutinin in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I). Values above were corrected by subtracting baseline proliferation levels.

Trial II resulted in no treatment effects prior to repletion (Figure 10). During the HGG challenge period (wk 9-15), however, proliferation levels were greater ($P < .007$) in the A-def lambs as compared to Con or A-rep lambs only on the first week of the challenge.

In Trial II vitamin A-deficiency resulted in a dramatic increase in peripheral blood lymphocyte proliferation in response to optimal concentrations of PHA. A 2 wk repletion was sufficient to decrease A-def lambs proliferation levels down to Con values. Elevated proliferation levels in response to PHA were also observed in investigations on A-def New Zealand Black mice (Gershwin et al., 1984). This strain of mice is highly susceptible to autoimmune disease and with prolonged vitamin A deficiency PHA-stimulated lymphocyte proliferation was increased. These observations suggest that vitamin A deficiency may result in a shift in T-cell population toward more helper/effector cells. Subsets of T-cells responsive to PHA are generally associated with helper/effector type functions while those responsive to ConA are generally associated with suppressor activities. The increased ConA-stimulated lymphocyte proliferation also observed in Trial II may represent a heightened state of suppression necessary to compensate for the elevated helper activity.

Ovalbumin (1 and .1 $mg \cdot ml^{-1}$) stimulated lymphocyte

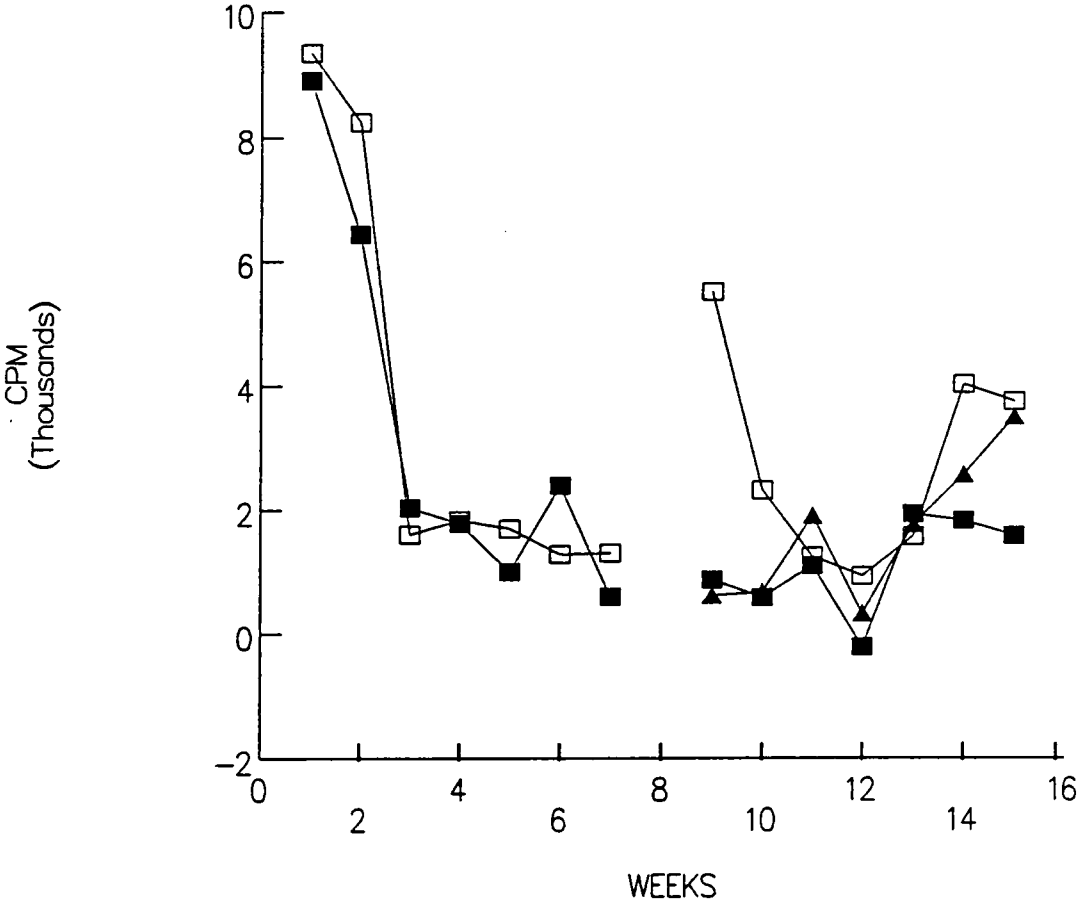

Figure 10. Lymphocyte proliferation in response to a 1:500 dilution of phytohemaglutinin in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II). Values above were corrected by subtracting baseline proliferation levels.

proliferation was unaffected by treatment in both Trials I (wk 1-7) and II (wk 1-7) (Figures 11, 12, 13, and 14). All treatment groups responded with increased lymphocyte proliferations in response to the primary challenge of ovalbumin. By the end of the ovalbumin challenge period there appeared to be a decline in ovalbumin-stimulated lymphocyte proliferation. This may be the result of spleen sequestering of the antigen-specific cells and thus removed them from the circulation. Lysozyme (wk 4-10) stimulated lymphocyte proliferation (Figure 11 and 13) was also not different between treatments for both lysozyme concentrations.

In Trial I HGG (wk 12-18) was ineffective in stimulating lymphocyte proliferation in either treatment. This lack of response was observed at both the high (Figure 11) and low (Figure 13) antigen concentrations. It is possible that HGG is not as immunogenic to lambs as ovalbumin. In Trial II (wk 9-15) HGG-stimulated lymphocyte proliferation was again much lower than when stimulated with ovalbumin (Figures 12 and 14). As in Trial I, HGG-stimulated lymphocyte proliferation of the Con and A-rep lambs in Trial II was not pronounced. Human gamma globulin-stimulated lymphocyte proliferation at both antigen concentrations, although quite low, was observed in the A-def lambs. At HGG concentrations of 1 $mg \cdot ml^{-1}$ (Figure 12)

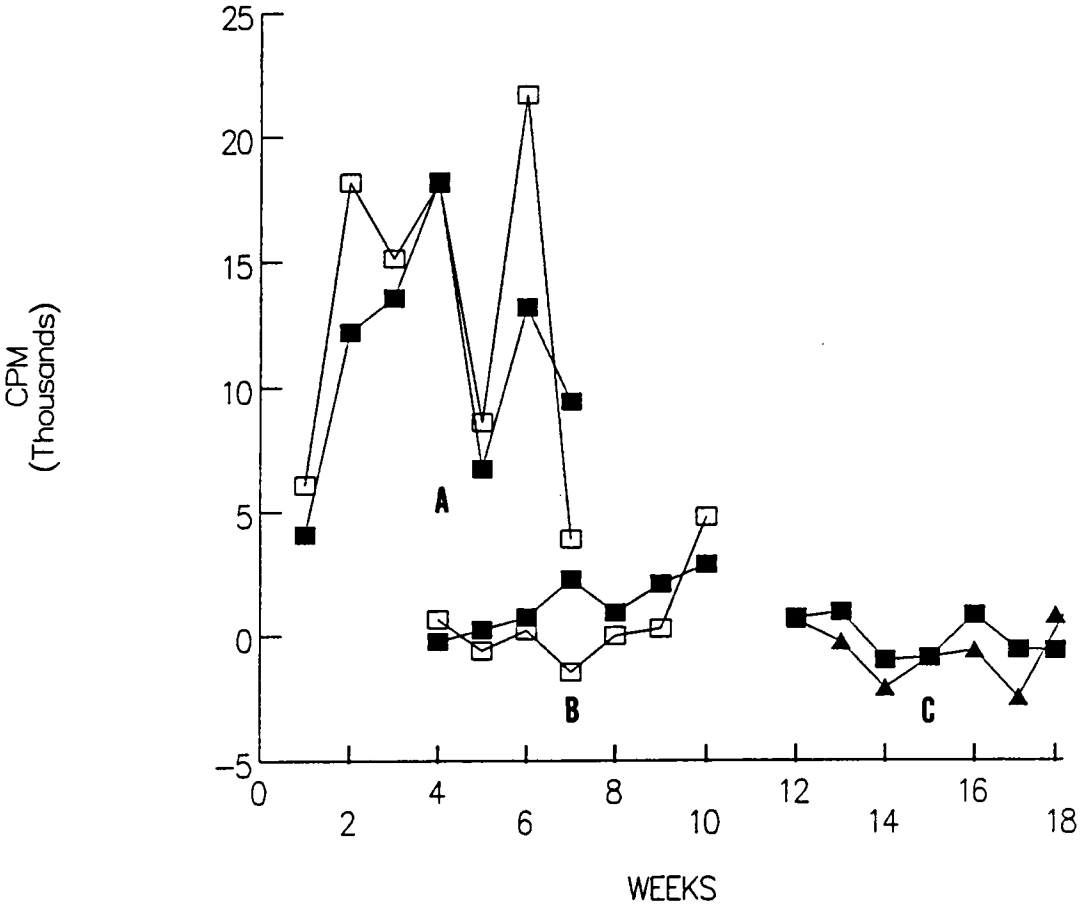

Figure 11. Lymphocyte proliferation in response to 1 $mg \cdot ml^{-1}$ of ovalbumin (**A**), lysozyme (**B**) and human gamma globulin (**C**) in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I).
Values above were corrected by subtracting baseline proliferation levels.

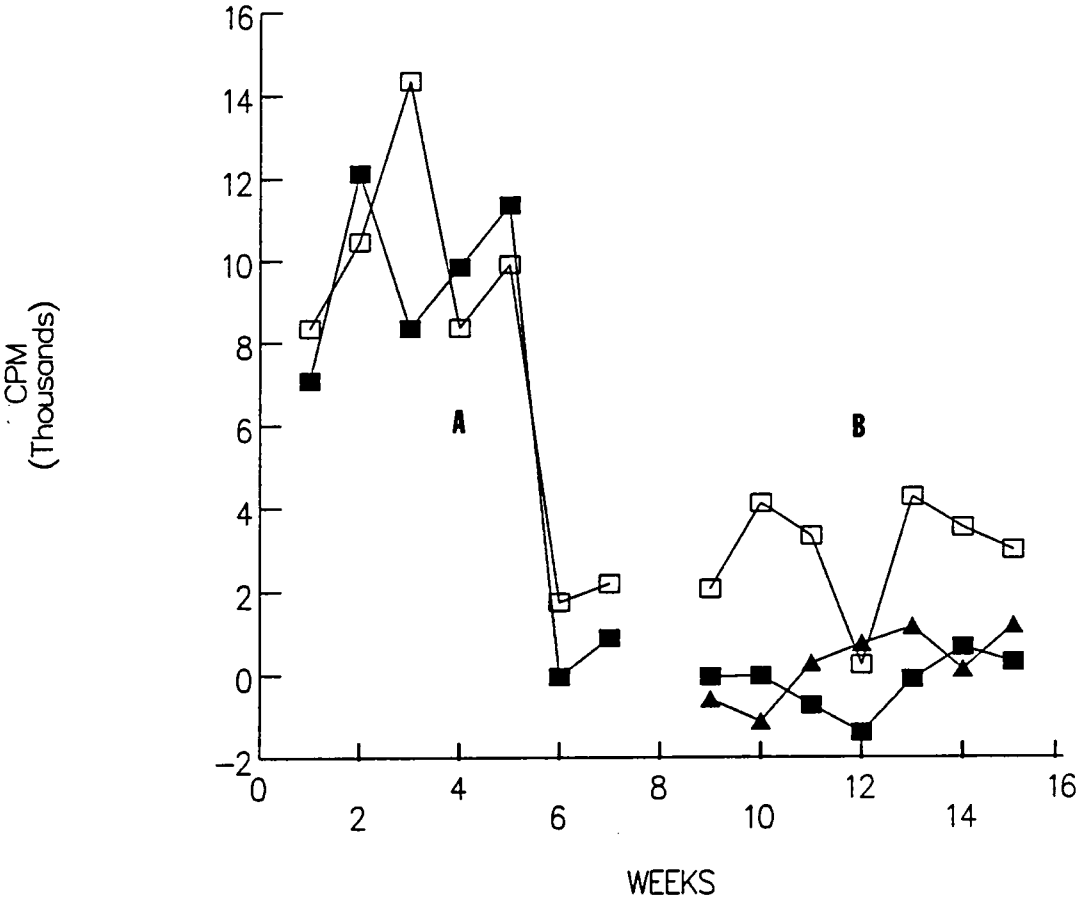

Figure 12. Lymphocyte proliferation in response to 1 $mg \cdot ml^{-1}$ of ovalbumin (**A**) and human gamma globulin (**B**) in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II). Values above were corrected by subtracting baseline proliferation levels.

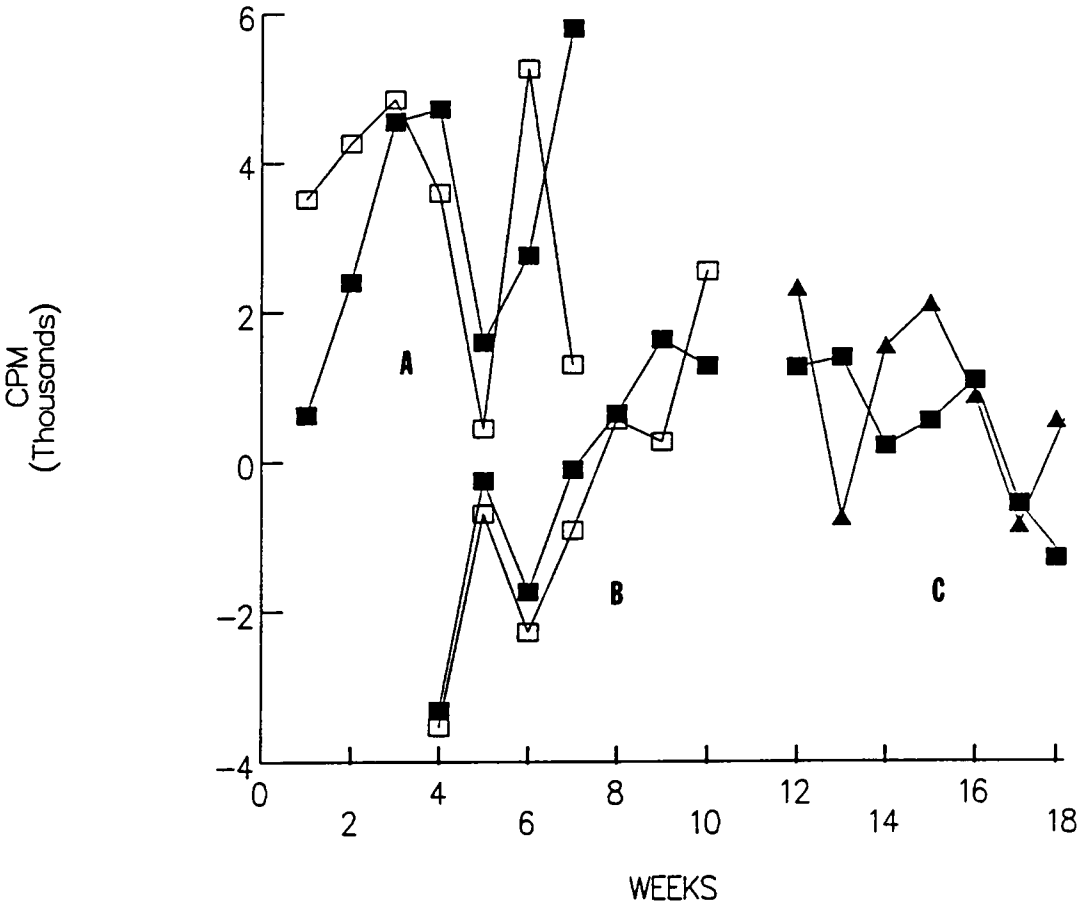

Figure 13. Lymphocyte proliferation in response to .1 $mg \cdot ml^{-1}$ of ovalbumin (**A**), lysozyme (**B**) and human gamma globulin (**C**) in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I). Values above were corrected by subtracting baseline proliferation levels.

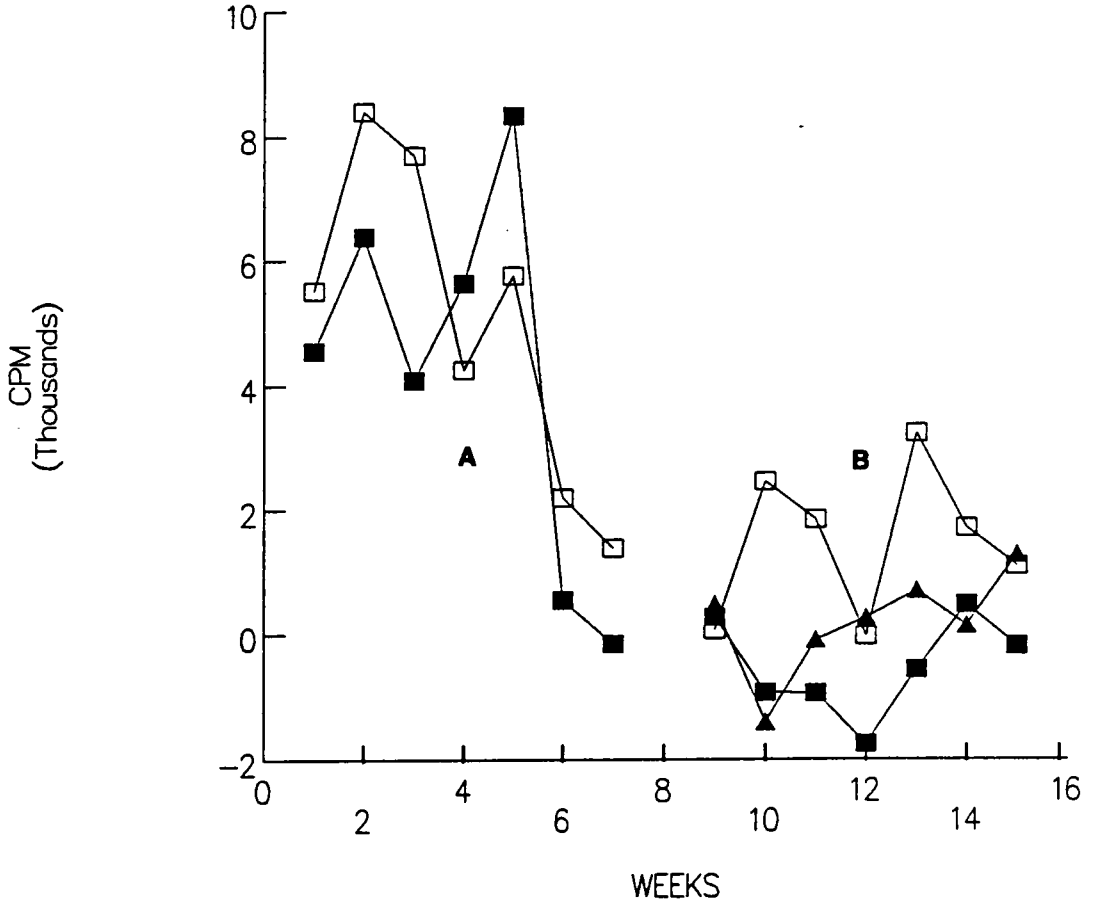

Figure 14. Lymphocyte proliferation in response to .1 mg·ml^{-1} of ovalbumin (**A**) and human gamma globulin (**B**) in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II). Values above were corrected by subtracting baseline proliferation levels.

A-def lymphocyte proliferation was greater ($P < .06$) than Con or A-rep proliferations on the first 3 wk of the challenge period. Human gamma globulin-stimulated lymphocyte proliferation was also greater ($P < .07$) in the A-def lambs on wk 13 and numerically greater on wk 14 and 15. At HGG concentrations of .1 $mg \cdot ml^{-1}$ (Figure 14) lymphocyte proliferations were similar to the higher HGG concentrations. Lymphocyte proliferation in response to .1 $mg \cdot ml^{-1}$ HGG was greater ($P < .02$)in the A-def lambs 1 wk after the primary challenge (wk 10) and 1 wk after the secondary challenge (wk 13) ($P < .05$). The observation that A-def lambs in Trial II had greater HGG-stimulated lymphocyte proliferation than Con or A-rep lambs is quite interesting. This increased responsiveness may be associated with the increased responsiveness of these same animals to PHA stimulation which was also observed. A shift in T-cell subset populations towards more helper/effector cells and less suppressor cells may account for the observed differences. The ratio of ccpm for optimal ConA stimulation to optimal PHA stimulation (suppressors/helpers) tended to be lower in the A-def lambs. Ratios were 2.98, 2.51 and 1.84 (+/- .31) for Con, A-rep and A-def lambs, respectively. In an associated report, the polyclonal serum IgG levels were higher in A-def lambs suggesting greater generalized immunologic

activity. Possible greater antigenic exposure due to epithelial degeneration and(or) greater overall T-helper cell activity is involved.

Serum cortisol concentrations fluctuated from week to week but were unaffected by treatment in both trials (Figures 15 and 16). There may have been a tendency for the A-rep lambs in both trials to have lower serum cortisol concentrations than Con lambs. This possible difference may be as much a consequence of increasing cortisol concentrations in the Con lambs as decreasing concentrations in the A-rep lambs. In Trial I serum cortisol concentrations in the A-rep lambs were lower than Con on wk 14 ($P < .04$) and 17 ($P < .11$) while differences between Con and A-rep lambs in Trial II were apparent on wk 16 ($P < .03$). The pattern of serum cortisol concentrations did not seem to have any particular relationship to observed differences in the present study in cell-mediated immune function or in humoral immune function (Chapter 4).

In summary, vitamin A-deficiency during the HGG challenge periods in Trial II resulted in increased lymphocyte proliferation to optimal suboptimal concentrations of ConA and optimal concentrations of PHA. Antigen-stimulated lymphocyte proliferation in the A-def lambs was also increased during this same period. A 2-wk vitamin A repletion was sufficient to return lymphocyte

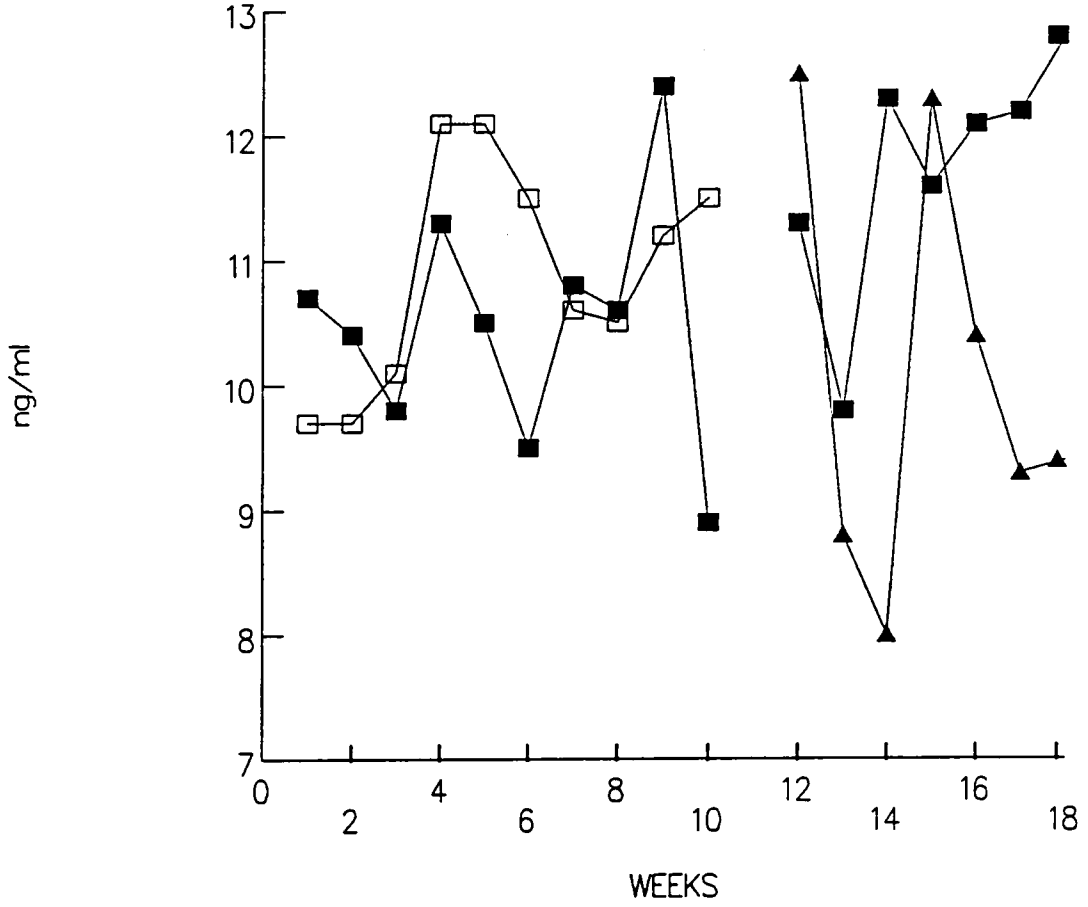

Figure 15. Serum cortisol concentrations in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial I).

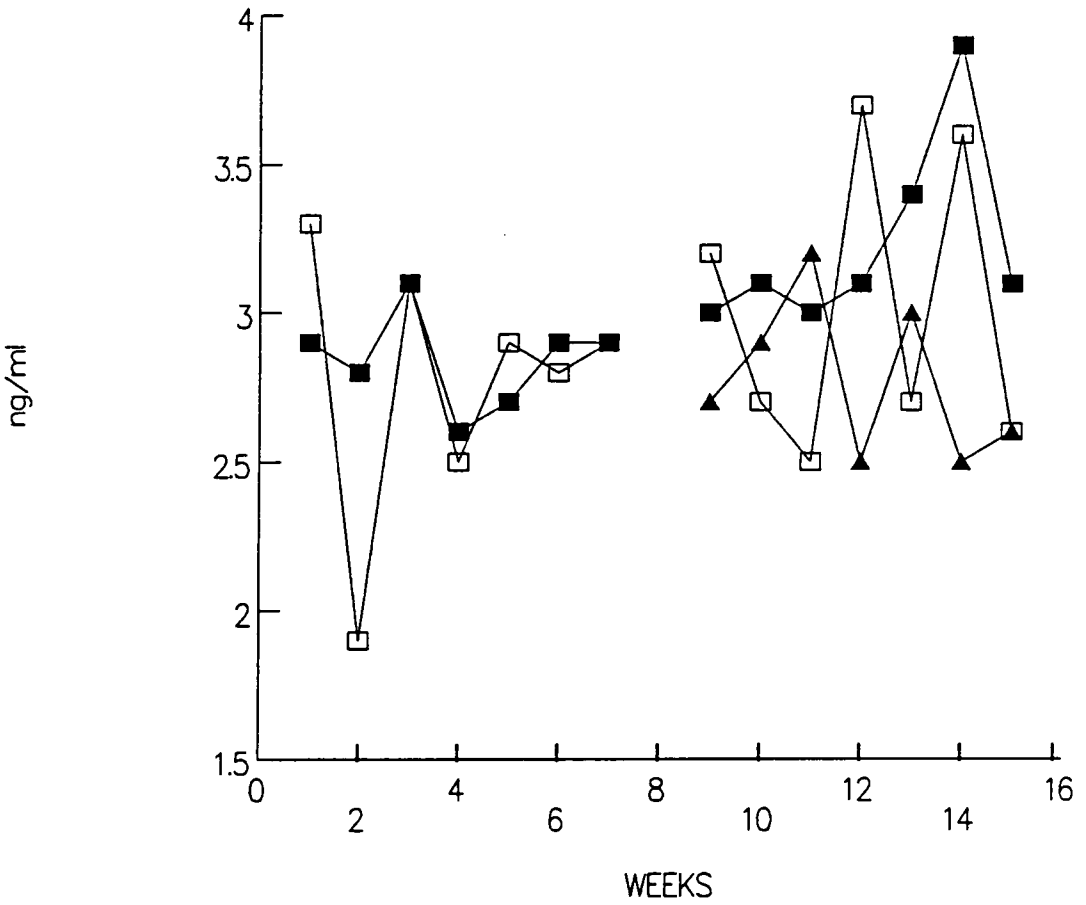

Figure 16. Serum cortisol concentrations in control (■), A-deficient (□) and A-repleted (▲) lambs (Trial II).

proliferation levels of A-def lambs to Con levels for all mitogen or antigen-stimulated proliferations. The data show vitamin A deficiency often increases the in vitro responsiveness of peripheral blood lymphocytes to various antigens and mitogens. Whether these responses are biologically significant in vivo and whether such responses help or hinder the lambs ability to ellicit a protective humoral and(or) cellular immunologic response remains to be answered. Serum cortisol concentrations in this study did not appear to mediate any observed changes in immunologic parameters.

Chapter VI

General Discussion

In both Trials I and II A-def lambs had reduced antigen-specific IgG concentrations in response to the secondary challenges. A secondary response, also referred to as an anemnestic response, is characterized by a rapid production of antibodies primarily of the IgG class. The rapid production of IgG is mediated by memory cells which are produced during the primary response. Any negative effect on memory cell production during the primary response would only be observed in the secondary response. Therefore, a decrease in the antigen-specific antibody production during the secondary response may be associated with problems that occurred in the primary response. The secondary response is also associated with a much greater production of IgG than in the primary response. Any slight impairment in antibody production especially IgG may not be observed in the primary response. The primary response is mediated mostly by IgM which is the major class of antibody produced.

Smaller antigen dosages seem to more greatly expose any nutritional effects observed in antigen-specific antibody production. These smaller antigen dosages are also

more physiologic and may more clearly delineate what is actually happening in vivo. Not only do doses of antigen effect the way animals respond with increased antibody production but the use of adjuvants can also be a factor. Adjuvants are used to more greatly stimulate the immune system. The most common adjuvant, Freund's incomplete adjuvant, is an oil based substance which functions to slowly release the injected antigen into the circulation. The antibody response obtained from the use of an oil based adjuvant could be quite different from that of an antigen injected with saline. The greater ease at which treatment effects were observed in antigen-specific antibody production in Trial II may be a direct result of antigen dosage and route of administration. Antigen dosages in Trial II were 1/50 (ovalbumin) and 1/5 (HGG) the dosages given in Trial I. Secondary injections were also given without Freund's incomplete adjuvant in Trial II which would further decrease the immunogenicity of the challenge. One must be cautious, however, when comparing the two trials in this experiment as effects due to dosage and route of administration are completely confounded with sex. It is the authors opinion, however, that antigen dosage probably played a major role in the treatment differences seen in antigen-specific antibody production between Trial I and II. Further research is needed to elucidate the

minimum dosage and proper route of administration required to more adequately expose any nutritional effects associated with humoral immunity.

The ability of a 2-wk vitamin A repletion period to reverse the immunosuppressive effects of the vitamin A deficiency varied between trials. Repletion in Trial I returned antigen-specific antibody production in the A-rep lambs to Con levels. In Trial II, however, the vitamin A-repletion failed to return antigen-specific antibody production to Con levels. Both the A-rep and A-def lambs had a decrease in antigen-specific antibody production as compared to Con values. There are three possible explanations for the differences between trials. Sex differences again can not be ruled out as the cause of these differences. A second plausible explanation deals with the amount of vitamin A supplementation given to the A-rep lambs. Vitamin A-repleted lambs were treated similarly during the 2-wk repletion period. Following repletion the lambs in Trial I were given 100,000 IU of vitamin A every 2 wk above what they received in the diet. In Trial II it was felt that this added supplementation was not necessary and A-rep lambs, therefore, only received the vitamin A contained in the diet. Vitamin A-repletion in Trial II was still adequate enough to return the A-def lambs serum vitamin A concentrations to Con levels after

the 2-wk repletion period. The third explanation for the difference between antigen-specific antibody production in the repletion periods between the two trials again may be related to antigen dosage. As mentioned previously, lower antigen dosages or the use of antigens with lower immunogenicities are more effective in elucidating nutritional effects on immune function. The antigen dose and route of administration used in the HGG challenge periods in Trial I may have masked any possible nutritional effects.

The inability of a 2-wk repletion period to restore proper antigen-specific antibody production to the A-def lambs in Trial II brings up some interesting questions. Serum vitamin A concentrations in the A-rep lambs in Trial II were similar to those of the Con lambs and yet antibody production was impaired. This implies that normal concentrations of vitamin A in the serum may not be enough to insure proper immune function. It may also imply that there is a lag phase between the time of vitamin A-repletion and the restoration of proper immune function. This lag phase must then be greater than 2 wk. One possible explanation for a lag phase may involve vitamin A's effects on proper maintainence of epithelial tissue. Peripheral lymph organs such as lymph nodes and the spleen are in part composed of epithelial tissue. If a vitamin A deficiency

impaired the function of the peripheral lymph organs by affecting proper epithelialization, immune function would be disturbed. Immune function would then only return to normal after proper epithelialization was restored. The time necessary for vitamin A-deficient tissue to return to normal after vitamin A-repletion may constitute the possible lag phase. Another possible explanation may be associated with vitamin A's effects on proper immune cell development. If a vitamin A-deficiency resulted in the impairment of immune cell development then a repletion of vitamin A would not show its affect until the defective cell population could be replaced by normal functioning cells. The observation that a 2-wk repletion period was unable to restore proper antigen-specific antibody production in Trial II brings up many unanswered questions.

Treatment differences in polyclonal serum IgG concentrations were apparent only during the HGG challenge periods in Trial II. At that time the A-def lambs had a greater concentration than either the Con or A-rep lambs. This finding is consistent with observations made previously in this laboratory involving vitamin A-deficient ewe lambs (Bruns, 1986). The elevated polyclonal serum IgG concentrations in the A-def lambs may be the result of a breakdown in the epithelial linings that make up the first line of defense against antigen invasions. An increased

rate of antigenic invasion could lead to elevated serum IgG concentrations. The A-rep lambs in both trials had polyclonal serum IgG concentrations similar to Con lambs. If it is true that the reason for increased serum IgG concentrations in the A-def lambs was due to a breakdown in the epithelial linings then it may be said that a 2-wk repletion of vitamin A was adequate to properly restore the epithelial linings. If one assumes that a 2-wk repletion period is sufficient for the regeneration of properly functioning epithelial linings, then the cause of impaired antigen-specific antibody production in the A-rep lambs must be do to some other factor (i.e. cell population shift). Other investigators have observed a greater incidence of autoimmune disease and a concurrent increase in serum polyclonal IgG concentrations in vitamin A-deficient mice (Gershwin et al., 1984). The elevated serum IgG concentrations observed in Trial II may have developed in response to self antigens. Further experimentation is necessary to more adequately understand the cause of elevated serum polyclonal IgG concentrations in A-def lambs.

Spleen wt in A-def lambs varied between Trial I and II. In Trial I, spleen wt tended to be only slightly higher than Con spleen wt. In Trial II spleen wt were dramatically larger in the A-def lambs as compared to Con or A-rep

lambs. A dramatic increase in spleen wt was observed previously in vitamin A-deficient ewe lambs by the author (Bruns, 1986). Repletion of A-def lambs returned spleen wt to Con values.

Liver wt also varied with between Trials I and II. Liver wt in Trial I tended to be slightly smaller in A-def lambs. In Trial II liver wt were dramatically larger in the A-def lambs. Control and A-rep lambs exhibited similar liver wt.

If we again assume that vitamin A deficiencies are associated with a breakdown of epithelial linings and a subsequent increase in infection rates the variation in organ wt can be explained. An increase in infection rate may result in an increase in wt of the organs which are responsible for filtering out antigens from the circulation. The spleen is a peripheral lymph organ which among other things is responsible for providing an environment suitable for the trapping of antigens and subsequent interactions between macrophages, T-cells and B-cells. The liver, too, is an organ which provides an environment for macrophages (Kupffer cells) who's functions are to filter out gastrointestinal antigens before they reach the peripheral circulation. A stage of deficiency resulting in a breakdown in the lining of the gastrointestinal tract would greatly increase the number of

antigens reaching the liver through the portal circulation. These antigens could be of food origin or consist of natural gastrointestinal flora. The increase in antigen numbers reaching the liver as a result of gastrointestinal epithelial breakdown could have an effect on that organs size.

The drop in PCV in the A-rep lambs during the HGG challenges in Trial I may have been the result of several factors. An increase in fluid volume during the HGG challenges may have diluted out the red blood cells and brought about a state of anemia. It is also possible that the anemia seen was hemolytic in origin. Gershwin et al., (1984) observed that mice prone to autoimmune disease developed this malady more quickly if the animals were on a vitamin A-deficient diet. The mice which developed the autoimmune disease on the vitamin A-deficient diet exhibited hemolytic anemia. It is possible that in Trial I the A-def lambs immune system was beginning to recognize self as foreign. This would have given rise to autoimmune T and B-cells. Even though the A-def lambs were repleted the autoimmune T and B-cell clones were still present and the hemolytic anemia persisted. This would also be supported by the fact that baseline lymphocyte proliferation rates observed during this same time were also increasing. Why then did this not occur in trial II? It may be that sex

differences played a key role in the differences. It is commonly observed that women are more susceptible to autoimmune diseases than men. If this holds true for other species of animals it could explain the differences in PCV between Trials I and II.

Peripheral blood lymphocyte proliferation in Trials I and II differed greatly when the cells were unstimulated. Trial I baseline proliferation values were greater throughout the ovalbumin and lysozyme challenge periods in the A-def lambs. Even after repletion the mean differences between the Con and A-rep lambs was evident. In Trial II differences in unstimulated peripheral blood lymphocyte proliferation were not observed between treatments. The variable responses observed between trials may be the result of sex differences but a more plausible explanation may involve the different sources of serum used in the culture medium. Something in the lamb serum which was used to culture the lymphocytes in Trial I may have been preferentially stimulating the A-def and A-rep lambs. This could imply that the A-def and A-rep lambs were recognizing something in the lamb serum not recognized by the Con lymphocytes. One could also conclude that the Con, A-def and A-rep lambs could all recognize or bind to this unknown substance(s) but the A-def and A-rep lambs had a greater affinity or greater number of receptors for this substance.

It is not known whether this substance is acting as a hormone or as an antigen (if it exists at all).

The discussion presented above illustrates an important question which needs to be addressed. When investigating the effects of nutrition on in vitro peripheral blood lymphocyte proliferation should one use autologous or heterologous serum in the culture medium. It is the author's opinion that not only should autologous serum be used but that the serum should be obtained from the individuals in each treatment and pooled for use in the culture of the respective treatment lymphocytes. Cell culture conditions are optimal conditions necessary for cell growth. Taking a cell out of the environment of the body and placing it into optimal cell culture conditions using heterologous serum is not physiologic. Any nutritional treatment differences in peripheral blood lymphocyte proliferation observed under these conditions are due to a defect in the cell population only. Cell culture in medium containing heterologous serum allows no interaction between cells and other soluble factors normally existing in the body. Although culturing peripheral blood lymphocytes in medium containing autologous serum (maybe more importantly, in serum from the treatment animals in question) may not clearly elucidate the factor or factors causing any observed treatment

differences it would be a more physiologic approach to identifying nutritional treatment differences.

Concanavalin A-stimulated lymphocyte proliferation was not different between treatments in either trial prior to repletion. Concanavalin A-stimulated peripheral blood lymphocyte proliferation of the A-rep lambs in both trials did not differ from controls. The A-def lambs in Trial II during the HGG challenge periods had a greater ConA-stimulated lymphocyte proliferation than either the Con or A-rep lambs. This observed elevation in ConA-stimulated lymphocyte proliferation is not consistent with observations made in mice (Nauss et al., 1979). Species differences and differences in the severity of the deficiency may be two possible explanations for the differences observed from previous investigations involving mice. Treatment differences in response to suboptimal PHA concentrations were observed prior to repletion in Trial I. Vitamin A-deficient lambs exhibited decreased proliferation in response to suboptimal PHA concentrations. Repletion of the A-def lambs resulted in no treatment differences. No treatment differences were observed in response to suboptimal PHA concentrations in Trial II. At optimal PHA concentrations treatment differences were observed only during the HGG challenge periods in Trial II. During this challenge period the A-def lambs exhibited a greater

proliferation rate than either the Con or A-rep lambs. This elevated PHA-stimulated peripheral blood lymphocyte proliferation is consistent with investigations involving vitamin A-deficient New Zealand Black mice (NZB) (Gershwin et al., 1984). This strain of mice is susceptible to autoimmune disease and is used as a model for the study of systemic lupus erythrematosus. No differences were observed in ovalbumin-stimulated peripheral blood lymphocyte proliferation in either trial. Human gamma globulin-stimulated lymphocyte proliferation, however, was greater in the A-def lambs as compared to Con or A-rep lambs in Trial II. If one assumes that vitamin A-deficient lambs act similarly to NZB mice an explanation for the elevated HGG lymphocyte proliferation may exist. It is possible that lambs on the A-def diet for an extended period of time could develop T-cell clones which recognize lamb gamma globulins. This would sensitize the clones to lamb gamma globulin and HGG may be similar enough to crossreact and further elicit a response in vitro. Although facts to back up this theory are not present the possibility does exist.

Cortisol concentrations in both trials varied weekly and this variation was probably the result of handling differences. The animals were bled via venapuncture and were thus stressed at the time of sample collection. The cortisol values obtained, therefore, may be more a

reflection of responses to a stressor than to physiologic circulating levels. In either respect there was a tendency for the serum cortisol concentrations of the A-rep lambs to be lower than Con values. This treatment difference in cortisol concentrations may be as much a consequence of increased cortisol concentrations in the Con lambs as decreased concentrations in the A-rep lambs. It is interesting to note, however, that serum cortisol concentrations and baseline lymphocyte proliferation during the HGG challenge period in Trial II seem to be negatively related in the A-def lambs. Every time cortisol concentrations fell, unstimulated lymphocyte proliferation rose and vice-versa. The relationship between cortisol and baseline lymphocyte proliferation in the A-rep lambs seems to be positively related. Every time cortisol concentrations rose so do the baseline lymphocyte proliferation rates. The trends, however, may be just a coincidence.

It is interesting to note how closely some of the observations made in the two trials run in this lab compare to previous work done with vitamin A-deficient NZB mice. Some of the observations made by Gershwin et al. (1984) in their experiment with A-def NZB include; increased spleen wt in A-def mice, increased baseline and PHA-stimulated lymphocyte proliferation, decreased PCV in A-def mice and

an elevated polyclonal IgG concentration in the A-def mice. Although not all of the above observations were made in both trials, similarities between A-def NZB mice and A-def lambs should not be overlooked.

The interaction between nutrition and immune function is enormously complicated. As the wealth of knowledge increases in the areas of nutrition and immunology so to will the knowledge of the interactions between these two disciplines. An interdisciplinary approach to the study of the interaction between nutrition and immune function is a logical course of action. A consorted effort on the part of both the nutritionist and immunologist is needed to properly begin to understand this research area.

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APPENDIX A

Antigen-Specific Serum Immunoglobulin G Determination

For the determination of immunoglobulin G (IgG) specific for ovalbumin, lysozyme and human gamma globulin (HGG) Nunc elisa plates[1] are coated overnight at 5 °C with 100 ul of a coating buffer containing 5 $ug \cdot ml^{-1}$ of the appropriate antigen (Ag). The plates are then washed four times with washing/incubation buffer using a Dynatech plate washer[2]. One hundred microliters of a solution containing .3% gelatin in coating buffer is then added to the wells and incubated for 1 h at 37 °C. The plates are again washed (3X) using the plate washer with the washing/incubation buffer. Serum samples are then serially diluted down the plates. The final volume of the serially diluted serum samples is 50 ul. Starting dilutions for ovalbumin, lysozyme and HGG are 1/25 or 1/4 depending on the concentration of antibody in the serum. A serum sample containing no ovalbumin, lysozyme or HGG Ab is used as the negative control. This could be the serum obtained just prior to the Ag challenge. Once the serially diluted serum

[1]Thomas Scientific, Swedesboro, NJ 08085 Cat. no. 6106L10.
[2]Dynatech Labotatories, Inc., Alexandria VA 22314.

samples are in the wells, the plates are again allowed to incubate at 37 °C for 1 h. The plates are washed as before (4X) and 100 ul of a solution containing peroxidase labelled rabbit anti-sheep IgG (Fc)[3] is added. The labelled Ab solution is diluted with phosphate buffered saline to a final dilution of 1:5000. The plates are incubated at 37 °C for 1 h and then washed as before (4X). A .1 mmol solution of 2,2-azinobis(3-ethylbez-thiazoline sulfonic acid) (ABTS) is then added to the wells (100 ul) and allowed to incubate at room temperature for 30 min in the dark. A greenish-blue color appears in the wells at this time. The reaction is stopped by adding 5% sodium dodecyl sulfate solution to each well (100 ul). The optical desities of each well are then measured using a Flow Titertek MCC/340 plate reader[4]. A titer is realized when the optical density of a given dilution is equal to that of the negative control.

Buffers

Coating Buffer: sodium carbonate buffer, pH 9.6
(for coating microtiter plate with antigen or antibody)

1.59 g Na_2CO_3
2.93 g $NaHCO_3$
.1 g Thimerosal
Make up to 1000ml with distilled water.

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[3]Pel-Freez Biologicals, Rogers, AR 72757.
Cat. no. 719008-1.
[4]Flow Laboratories Inc. McLean, VA 22102.

Blocking Buffer: use the sodium carbonate buffer above substituting .3% gelatin for the antigen.

Washing/Incubation Buffer: PBS/Tween buffer, pH 9.8

8.0 g NaCl
.2 g KH_2PO_4
1.15 g Na_2HPO_4
.2 g KCl
.5 ml Tween 20
.1 g Thimerosal
Make up to 1000ml with distilled water.

ABTS substrate for Peroxidase:

55 mg ABTS in 100 ml .1M Sodium Citrate pH 4.2.
This solution is stable for 1 wk at 4 °C and 4 mo at -20 °C. Immediately before use add 1 ul 30% H_2O_2 per ml of the ABTS solution. Discard any unused ABTS-H_2O_2 solution.

Phosphate Buffered Saline: pH 7.6

2.84 g Na_2HPO_4
14.61 g NaCl
Make up to 1000 ml with distilled water.

APPENDIX B

Serum Polyclonal Immunoglobulin G Determination

Nunc elisa plates are coated overnight at 5 °C with 100 ul of a coating buffer containing 5 $ug \cdot ml^{-1}$ rabbit anti-sheep IgG (Fc)[1]. The plates are washed (4X) with a plate washer using washing/incubation buffer. The plates are then incubated for 1 h at 37 °C with 100 ul of .3% gelatin coating solution to block nonspecific binding sites. The plates are washed (3X) as before and a 1:100,000 dilution of serum is then pipetted into triplicate wells (50 ul). Standards (40, 20, 10, 5, 2.5, and 1.25 $mg \cdot ml^{-1}$) are also diluted 1:100,000 and pipetted (50 ul) with each plate. Both the serum samples and standards are diluted with washing/incubation buffer. The plates are then incubated for 1 h at 37 °C and then washed (4X). The second antibody solution (peroxidase labelled) is then added to each well and allowed to incubate as described in appendix A. Following the 1 h incubation period 100 ul of the substrate ABTS is added to each well and allowed to incubate in the dark for 20 min. The reaction is stopped with 5% sodium dodecyl sulfate and optical densities are then obtained using the Flow Titertek MCC/340 plate reader.

[1]Pel-Freez Biologicals Rogers, AR 72756 Cat. no. 719002-1.

Concentrations are obtained by utilizing the Skan Soft I software package supplied by Flow laboratories. Briefly, the plate reader is connected to an IBM compatible PC. With the use of the Skan Soft I package optical densities are determined for unknowns and standards. The computer calculates a standard curve using a log-linear transformation and unknown concentrations are determined from this standard curve.

The buffers used in this assay are the same buffers used in the antigen-specific IgG assay presented in Appendix A.

APPENDIX C

Peripheral Blood Lymphocyte Proliferation

The culturing and subsequent measurement of lymphocyte proliferation must be performed in a sterile environment. The assay must be performed in a laminar flow hood and hands must be washed with 70% ethyl alcohol each time they enter the hood. All tubes, pipettes, pipette tips and any other material which may come in contact with the lymphocytes must be autoclaved. All solutions or buffers used in the assay must also be sterile either by autoclaving or sterile filtering. The maintainence of sterility throughout the assay is of great importance.

Ten milliliters of whole blood are mixed with 10 ml of hank's balanced salt solution (HBSS) and layered over 10 ml of Ficoll-hypaque[1] in a 50 ml sterile plastic centrifuge tube. One could also use a buffy coat instead of whole blood as a source of lymphocytes. In that case a heparinized blood sample is spun at 500 X G at 5 °C for 20 min. The buffy coat which forms on the top of the red blood cells (RBC) is harvested with a sterile 5 ml pipette and mixed with 5 ml HBSS. This mixture is then layered over 10 ml Ficoll-hypaque. The procedure from this point on is the

[1]Sigma Diagnostics St. Louis, MO 63178 Cat. no. 1077-1.

same if using either whole blood or the buffy coat. The tubes are centrifuged at room temperature for 30 min at 400 X G with the brake off. A sterile pasteur pipette is then used to remove the layer of white blood cells (WBC) which are located on the ficoll layer. The WBC are placed in a 10 ml sterile tube and centrifuged for 10 min at 250 X G (Brake on). The supernatant is then poured off leaving the pellet. The pellet formed may contain some RBC. To remove the RBC the pellet is resuspended with 1 ml of a tris buffer warmed to 37 °C. The cells are incubated for 10 min at 37 °C and then 5 ml of HBSS is added to each tube. The tubes are again centrifuged for 10 min at 250 X G and the supernatant is again decanted. If RBC still exist the cells are again treated with tris buffer in the aforementioned procedure. The cells should not be treated with tris more than twice. After the last tris treatment the cells are washed twice in HBSS and pelleted at 250 X G. The use of a buffy coat as a source of lymphocytes results in little RBC contamination and therefore the use of the tris buffer for the lysis of RBC may be unnecessary.

The WBC pellet is resuspended in 1 ml media. A tenth of a milliliter of this cell suspension is added to .1 ml trypan blue[2] and .8 ml of HBSS. This solution is put on a hemacytometer where the cells are counted. After the number

[2]Sigma Diagnostics St. Louis, MO 63178 Cat. no. T 9520.

of cells has been determined, an apropriate amount of media is added to the remaining .9 ml cell suspension to produce a concentration of 1 X 10^6 cells per ml. One hundred microliters of this cell suspension is added to each well in a sterile cell culture plate. One hundred microliters of either concanavalin A (ConA), phytohemaglutinin (PHA) or the antigens used during the challenge period are added to the 100 ul cell suspension in triplicate. The final concentrations of mitogens are 16 and 1.6 $ug \cdot ml^{-1}$ for ConA and a 1:10 (1:20 Trial II) and 1:500 dilution of PHA. The Ag are added to the wells at concentrations of 1 and .1 $mg \cdot ml^{-1}$. Three wells containing 100 ul of media are used as control wells to measure baseline lymphocyte proliferation rates. The mitogen-stimulated cells are incubated at 37 °C in 5% CO_2 for 72 h while the antigen stimulated cells are incubated for 120 h. Upon completion of the incubation period, the wells are pulsed with 1 uCi of tritiated thymidine per well. The plates are again incubated at 37 °C in 5% CO_2 for 16 h at which time the cells are harvested with a Mini-Mash II cell harvester[3]. The filter paper containing the tritiated DNA is placed in a 7 ml scintilation vial and 3 ml of scintilation cocktail is added. The vials are counted on a beta counter for 2 min each.

[3]Whittaker M.A. Bioproducts Walkersville, MD 21793.

Reagents

Hank's balanced salt solution:

Hank's balanced salt solution (10 X)[4]	10.0 ml
Sodium Bicarbonate[5]	.5 ml
Hepes[6]	.5 ml
Penicillin-Streptomycin[7]	.5 ml
Sterile Water	88.5 ml
	100.0 ml

Media:

Lamb Serum[8] (Trial I) (Heat inactivated)[9]	10.0 ml
or	
Fetal calf serum10 (Trial II) (Heat inactivated)[9]	10.0 ml
Amino acid-sodium pyruvate[11]	1.5 ml
L-glutamine[12]	1.0 ml
Hepes[6]	.5 ml
2-mercaptoethanol[13]	.1 ml
RPMI 1640[14]	83.9 ml
	100.0 ml

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[4]Gibco Laboratories Grand Island, NY 14702 Cat. no. 310-4180AJ.
[5]Sigma Diagnostics St. Louis, MO 63178 Cat. no. S 8761.
[6]Sigma Diagnostics St. Louis, MO 63178 Cat. no. H 0887.
[7]Sigma Diagnostics St. Louis, MO 63178 Cat. no. P 3539.
[8]KC Biologicals Lenexa, KS 66215 Cat. no. 3004
[9]Heat inactivation: heat serum to 56 °C for 30 min.
[10]KC Biologicals Lenexa, KS 66215 Cat. no. 12-103.
[11]Sodium pyruvate (.5g) is disolved in 50 ml of distilled water and then sterile filtered. One milliliter of this solution is added to 2 ml of the amino acid solution (Sigma Cat. no. M 7145).
[12]KC Biologicals Lenexa, KS 66215 Cat. no. LM-233-1.
[13]5 X 10^{-2} M.
[14]Gibco Laboratories Grand Island, NY 14702 Cat. no. 320-1870PJ.

Tris Buffer:

NH_4Cl (.16 M)	90 ml	
Tris base (.17 M)	10 ml	
	100 ml	pH 7.2

Tritiated pulse solution:

Tritiated thymidine (6.7 $Ci \cdot mmol^{-1}$)	.5 ml
RPMI 1640	4.5 ml
	5.0 ml

Pulse each well with 10 ul of the above solution.

APPENDIX D

Serum Vitamin A Analysis

Separation. To a 20 ml centrifuge tube was added 5.0 ml of serum which had been allowed to reach room temperature. Five milliliters of ethanol were added to the serum and the mixture was cooled in a ice bath. To the serum-ethanol mixture was added 5.0 ml of petroleum ether at which time the tubes were tightly capped. The rack of tubes was placed on its side on a horizontal shaker and shook for 5 min. The tubes were then centrifuged for 1 min to insure complete phase separation, and 4.0 ml of the ether layer (top layer) were carefully pipetted off and placed in colorimeter tubes. The ether was then evaporated to dryness under vacuum (approximately 6 h).

Color Development. After the ether was completely evaporated, 1.5 ml of chloroform were added to the colorimeter tubes to dissolve the residue remaining. Trifluoroacetic acid (1.5 ml) was then added to the chloroform and quickly but gently mixed. The colorimeter tube was then placed in a spectrophotometer[1] and percent transmittance read at 616 mu within 5 s from the time the trifluoroacetic acid was added.

[1]Bausch and Lamb, Spectronic 20.

From the time that 4.0 ml of the petroleum ether layer were pipetted into the colorimeter tubes until the percent transmittance was read on the spectrophotometer, care was taken to reduce the amount of light which struck the tubes. This was done by shutting off all of the lights in the lab and cracking the outside door so one could see well enough to work. The extraction phase of the vitamin A assay is a modification of the Kimble (1939) procedure while the color development phase is the procedure of Dugan et al. (1964).

Vitamin A concentrations were calculated by converting percent transmittance to optical density and then multiplying by the exctinction coefficient (7.318) which was obtained from a previously prepared standard curve. To convert the concentration to $ug \cdot dl^{-1}$ the product obtained above is multiplied by 25.

APPENDIX E

Liver Vitamin A Analysis

Digestion. A liver sample core, 1 g wet weight, was digested in 10% alcoholic potassium hydroxide. This solution was made by dissolving 10 g potassium hydroxide in 20 ml of water followed by the addition of 80 ml of ethanol. Ten milliliters of this solution were added to the liver sample and heated in a hot water bath at 90 C until the liver was digested.

Extraction. To the digested liver samples was added 10 ml of water and 10.0 ml of petroleum ether. The tubes were tightly capped and shook on a horizontal shaker for 5 min. The tubes were spun to seperate the phases and 1.0 ml of the petroleum ether layer (top layer) was pipetted into a colorimeter tube. From this point the remainder of the assay is the same as that used to develop the color in the serum vitamin A assay. Calculations are also similar to that performed in the serum vitamin A assay.

APPENDIX F

Serum Cortisol Determination

Procedure. Standards[1] were serially diluted with 50 ul being pipetted into 12 X 75 mm borosilicate tubes. To these tubes was added 50 ul of deionized water. One hundred microliters of serum were pipetted into clean 12 X 75 mm borosilicate tubes and both the standard and serum unknown tubes were treated similarly through the remainder of the assay.

Cortisol ^{125}I derivative[1] (100 ul) was added to the tubes using a hamilton repeating syringe[2]. A cortisol antibody suspension[1] (100 ul) was then added, again using a hamilton repeating syringe. The tubes were then each mixed for 5 s on a vortex mixer, covered with plastic film and incubated in a water bath at 37 C for 1.5 h. After the incubation period, the tubes were centrifuged at room temperature for 15 min at 1500 x g.

The tubes were then placed in test tube racks which were equipped with double sided tape so that when the rack was turned up-side down the tubes would remain in the rack. The liquid was decanted in this way with the tubes being

[1]Amersham Cortisol RIA Kit, Amersham Corp., Arlington Heights, IL. 60005.
[2]Fischer Scientific, Raleigh, NC. 27604.

allowed to drain up-side down on absorbant paper for 10 min. After this time the racks were reinverted and the tubes placed in a gamma counter and counted.

Total count tubes were also counted in the gamma counter. These tubes contained only 100 ul of the cortisol ^{125}I derivative and were not involved in any of the other steps mentioned above.

Calculations. A log-logit plot was used to determine cortisol concentrations. The percent bound of the standards was plotted against their known concentrations on log-logit graph paper and the concentrations of the unknown samples were determined from this graph.

% Bound = $B/B_0 \times 100$

B = Sample counts

B_0 = Average zero standard counts

APPENDIX G

TABLES

TABLE 5. EFFECTS OF VITAMIN A STATUS ON OVALBUMIN-SPECIFIC SERUM IMMUNOGLOBULIN G CONCENTRATIONS

Trial I

Days	Treatment		SE[a]	Prob.[b]
	Control	A-deficient		
	-----Titer (log_2)------			
0	0	0	0	0
7	.3	.2	.16	.77
14	3.8	2.7	.54	.18
21	5.3	4.4	.55	.28
28	8.0	7.5	.42	.38
35	8.0	7.1	.40	.14
42	9.2	8.3	.36	.09

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 6. EFFECT OF VITAMIN A STATUS ON LYSOZYME-SPECIFIC SERUM IMMUNOGLOBULIN G CONCENTRATIONS

Trial I

Days	Treatment		SE[a]	Prob.[b]
	Control	A-deficient		
	-----Titer (log_2)-----			
21	0	0	0	0
28	.9	1.5	.34	.29
35	5.3	5.3	.79	.95
42	7.5	6.8	.75	.50
49	8.9	8.3	.61	.45
56	8.5	7.9	.55	.41
63	9.2	8.8	.46	.55

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 7. EFFECT OF VITAMIN A STATUS ON HUMAN GAMMA GLOBULIN-SPECIFIC SERUM IMMUNOGLOBULIN G CONCENTRATIONS

Trial I

Day	Treatment		SE[a]	Prob.[b]
	Control	A-repleted		
	------Titer (log_2)-----			
77	0	0	0	
84	2.1	2.0	.85	.94
91	6.9	7.4	.88	.70
98	7.2	8.2	.71	.35
105	8.5	9.2	.55	.39
113	8.5	8.5	.43	1.00
119	8.8	8.9	.48	.89

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 8. EFFECT OF VITAMIN A STATUS ON OVALBUMIN-SPECIFIC SERUM IMMUNOGLOBULIN G CONCENTRATIONS

Trial II

Day	Treatment		SE[a]	Prob.[b]
	Control	A-deficient		
	------Titer (log_2)------			
0	0	0	0	0
7	1.5	1.8	.30	.56
14	2.6	2.9	.27	.45
21	2.6	3.5	.33	.07
23	7.6	7.5	.28	.71
25	10.6	9.6	.37	.07
27	11.3	10.6	.30	.12
28	11.0	10.1	.33	.06
35	10.4	9.2	.37	.03
42	10.9	9.7	.33	.02

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 9. EFFECT OF VITAMIN A STATUS ON HUMAN GAMMA GLOBULIN-SPECIFIC SERUM IMMUNOGLOBULIN G CONCENTRATIONS

Trial II

	Treatment				Probability[a]		
Day	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	--------Titer (log_2)--------						
56	0	0	0				
63	4.6	4.1	3.8	.54	.58	.88	.31
70	7.8	6.2	7.2	.56	.15	.07	.46
77	7.6	6.6	7.1	.53	.42	.26	.51
85	8.4	7.7	8.0	.52	.64	.44	.59
92	9.0	9.1	8.4	.36	.36	.40	.25
94	9.9	9.0	8.7	.35	.06	.38	.01
96	9.7	9.0	8.8	.43	.31	.62	.15
98	8.2	7.3	7.5	.38	.23	.26	.19
99	9.5	8.1	8.2	.32	.01	.07	.01

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 10. EFFECT OF VITAMIN A STATUS ON SERUM POLYCLONAL IMMUNOGLOBULIN G CONCENTRATIONS

Trial I

Week	Treatment: Control	A-deficient	A-repleted	SE[a]	Prob.[b]
	mg·ml^{-1}				
1	16.2	18.7		3.1	.58
2	18.2	17.1		1.6	.65
3	15.1	15.1		1.5	.99
4	14.3	12.4		1.0	.23
5	12.4	13.1		1.3	.70
6	12.1	12.0		1.5	.98
7	12.9	13.3		1.6	.84
8	13.4	12.4		1.8	.70
9	11.6	12.1		1.2	.79
10	13.8	14.6		1.9	.78
11	REPLETION				
12	13.4		13.2	1.4	.94
13	13.1		12.2	1.5	.69
14	12.4		12.8	1.3	.81
15	12.5		13.7	1.2	.47
16	14.5		13.7	1.6	.72
17	13.3		14.9	1.7	.53
18	13.6		13.4	1.5	.90

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 11. EFFECT OF VITAMIN A STATUS ON SERUM POLYCLONAL IMMUNOGLOBULIN G CONCENTRATIONS

Trial II

	Treatment				Probability[a]		
Week	Control(1)	A-def(2)	A-rep(3)	SE[b]	TRT	1,3 vs 2	1 vs 3
	mg·ml^{-1}						
1	16.1	17.8		2.4	.62		
2	16.7	19.0		2.9	.59		
3	17.3	19.6		3.1	.61		
4	14.7	15.3		1.8	.81		
5	15.4	16.3		1.6	.67		
6	12.9	15.6		1.8	.31		
7	15.6	18.6		2.0	.31		
8	REPLETION						
9	21.6	27.7	22.7	3.5	.44	.21	.82
10	17.9	25.3	21.9	2.6	.16	.11	.29
11	17.0	27.7	18.3	3.0	.04	.01	.76
12	17.7	29.6	17.3	3.7	.05	.02	.94
13	15.1	22.8	15.1	2.2	.04	.01	.99
14	18.5	29.0	20.2	3.8	.16	.06	.74
15	18.7	27.5	19.6	2.4	.04	.01	.78

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 12. EFFECT OF VITAMIN A STATUS ON PACKED CELL VOLUME

Trial I

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	----------%	----------		
1	37.0	36.8	.74	.88
3	37.2	37.2	.78	.98
5	36.2	38.5	.76	.05
7	37.8	36.6	.79	.28
9	36.5	37.0	.71	.58
11	----------Repletion----------			
12	36.4	35.4	.96	.48
14	36.1	35.7	.68	.69
16	37.6	34.9	.82	.05
18	37.4	34.0	.85	.02

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 13. EFFECT OF VITAMIN A STATUS ON PACKED CELL VOLUME

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt 1,3 vs 2	1 vs 3	
	----------%						
1	33.7	35.2		.56	.08		
2	34.1	35.1		.62	.30		
3	33.5	34.8		.63	.18		
4	34.3	34.2		.58	.95		
5	30.1	31.2		.66	.29		
6	32.2	33.5		.46	.07		
7	32.9	34.2		.59	.14		
8	----------Repletion----------						
9	32.2	33.3	33.0	.83	.68	.52	.45
11	31.9	32.9	32.2	.89	.72	.45	.81
12	31.9	33.7	32.4	.85	.33	.16	.68
13	32.4	33.6	32.6	.72	.51	.26	.84
15	34.0	34.4	34.4	.76	.91	.82	.71

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 14. EFFECTS OF VITAMIN A STATUS ON UNSTIMULATED LYMPHOCYTE PROLIFERATION

Trial I

Week	Treatment: Control	A-deficient	A-repleted	SE[a]	Prob.[b]
	CPM X 10^{-3}				
1	17.3	20.1		2.90	.51
2	21.9	31.2		2.78	.03
3	25.2	29.2		3.04	.37
4	22.4	32.7		4.50	.13
5	13.5	19.3		1.41	.01
6	22.7	34.4		3.82	.08
7	12.2	16.0		2.90	.37
8	14.4	20.4		2.98	.17
9	20.0	28.7		3.86	.13
10	15.2	25.5		2.12	.004
11	REPLETION				
12	15.2		21.2	4.26	.34
13	15.0		24.4	5.24	.24
14	17.1		32.2	5.49	.08
15	13.5		30.6	6.10	.08
16	14.1		26.7	5.32	.11
17	20.0		40.3	7.65	.09
18	16.5		42.3	8.62	.06

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 15. EFFECTS OF VITAMIN A STATUS ON UNSTIMULATED LYMPHOCYTE PROLIFERATION

	Trial II						
	Treatment				Probability[a]		
WK	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	CPM X 10^{-3}						
1	4.1	3.5		.88	.62		
2	3.2	2.8		.54	.56		
3	1.5	2.5		.47	.15		
4	1.0	1.2		.26	.72		
5	1.4	1.3		.29	.95		
6	1.0	1.7		.29	.15		
7	1.5	1.3		.26	.54		
8	REPLETION						
9	1.6	2.3	1.3	.45	.29	.14	.58
10	1.9	3.2	2.3	.50	.21	.09	.59
11	2.1	4.7	2.9	1.05	.22	.10	.59
12	2.5	1.5	1.6	.81	.60	.58	.40
13	1.7	2.3	2.2	.45	.59	.54	.42
14	1.4	2.0	1.7	.33	.47	.31	.50
15	1.8	2.7	3.1	.53	.21	.75	.09

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 16. EFFECTS OF VITAMIN A STATUS ON CONCANAVALIN A (16 ug·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

Week	Treatment: Control	A-deficient	A-repleted	SE[a]	Prob.[b]
	CCPM[c] X 10^{-4}				
1	13.6	12.7		.88	.49
2	17.5	18.6		1.28	.55
3	17.5	15.3		1.16	.18
4	21.6	18.0		1.43	.09
5	12.7	11.1		.90	.22
6	17.7	15.9		1.19	.28
7	12.4	9.6		1.17	.11
8	12.4	11.3		.99	.43
9	19.8	17.3		1.47	.25
10	15.8	15.2		1.75	.73
11	REPLETION				
12	22.2		18.7	2.66	.38
13	18.0		14.1	1.38	.08
14	20.9		17.8	1.14	.09
15	9.1		8.5	1.11	.71
16	21.3		16.7	2.01	.21
17	12.2		10.3	2.72	.63
18	7.7		8.9	1.23	.51

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 17. EFFECT OF VITAMIN A STATUS ON CONCANAVALIN A (16 ug·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	---------CCPM[c] X 10^{-4}---------						
1	21.2	18.3		1.30	.14		
2	12.1	10.9		1.67	.64		
3	12.1	14.3		1.46	.30		
4	12.8	13.5		1.63	.79		
5	12.7	15.7		2.04	.32		
6	10.4	11.9		1.04	.33		
7	9.4	9.7		1.08	.89		
8	----------REPLETION----------						
9	8.9	15.0	7.7	1.99	.04	.01	.72
10	9.9	13.6	7.7	1.71	.07	.03	.39
11	11.4	16.2	13.4	1.89	.22	.12	.46
12	10.0	10.1	9.8	1.68	.99	.93	.92
13	10.6	16.8	15.6	1.64	.04	.09	.04
14	9.6	15.8	11.0	1.72	.06	.02	.55
15	13.8	18.5	14.4	1.63	.12	.04	.79

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 18. EFFECT OF VITAMIN A STATUS ON CONCANVALIN A (1.6 ug.ml-1) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

	Treatment				
WEEk	Control	A-def	A-rep	SE[a]	Prob.[b]
	-------CCPM[c] X 10^{-3}-------				
1	40.7	36.3		6.88	.65
2	47.7	56.3		6.06	.33
3	48.4	40.4		6.26	.38
4	57.9	50.0		6.81	.43
5	25.4	24.9		4.17	.93
6	37.4	42.0		5.27	.63
7	22.2	15.7		3.42	.20
8	17.7	16.5		2.72	.78
9	71.6	62.8		7.68	.43
10	32.6	37.6		5.83	.59
11	----------REPLETION----------				
12	52.4		34.5	13.65	.38
13	34.1		24.2	4.84	.18
14	38.5		26.9	6.61	.25
15	10.2		4.9	2.03	.09
16	35.6		25.2	5.76	.36
17	9.2		5.3	3.80	.48
18	5.1		5.4	2.05	.92

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 19. EFFECT OF VITAMIN A STATUS ON CONCANVALIN A (1.6 ug.ml-1) STIMULATED LYMPHOCYTE PROLIFERATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	----------CCPM[c] X 10^{-3}--------						
1	94.2	99.1		13.74	.81		
2	76.1	64.1		12.42	.51		
3	73.6	92.3		17.19	.46		
4	48.5	45.7		40.80	.86		
5	48.8	50.0		11.66	.94		
6	30.9	27.8		5.76	.71		
7	28.2	21.20		4.69	.31		
8	----------REPLETION----------						
9	30.7	68.2	18.7	10.63	.01	.004	.44
10	23.5	34.4	19.2	6.39	.25	.11	.64
11	29.1	51.7	29.9	8.16	.11	.04	.94
12	23.0	16.7	25.0	6.61	.66	.38	.83
13	40.8	73.7	53.1	14.10	.04	.09	.04
14	18.5	37.0	21.5	6.59	.15	.06	.74
15	32.2	46.7	32.5	7.37	.33	.14	.98

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 20. EFFECT OF VITAMIN A STATUS ON PHYTOHEMAGLUTININ (1:10) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

	Treatment				
Week	Control	A-def	A-rep	SE[a]	Prob.[b]
	---------CCPM[c] X 10^{-4}-------				
1	10.1	9.7		.90	.76
2	14.2	15.5		1.00	.36
3	14.8	16.7		.93	.18
4	18.2	14.3		.80	.004
5	9.9	7.2		.76	.03
6	15.6	13.5		1.29	.31
7	8.2	7.1		1.18	.50
8	11.7	9.6		1.06	.18
9	20.0	18.2		1.47	.42
10	14.0	14.7		1.23	.76
11	----------REPLETION----------				
12	21.6		19.7	2.25	.58
13	20.0		15.4	1.07	.01
14	22.2		21.4	1.38	.69
15	21.3		22.2	1.51	.71
16	21.8		20.6	1.33	.82
17	24.4		21.0	2.61	.39
18	18.1		19.8	1.65	.48

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 21. EFFECT OF VITAMIN A STATUS ON PHYTOHEMAGLUTININ (1:20) STIMULATED LYMPHOCYTE PROLIFERATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	CCPM[c] X 10^{-4}						
1	16.6	19.7		1.9	.27		
2	14.3	14.1		1.8	.94		
3	11.8	15.1		1.9	.23		
4	8.9	11.4		2.1	.41		
5	8.7	13.6		2.2	.13		
6	6.6	7.0		1.4	.86		
7	4.5	5.5		.9	.45		
8	REPLETION						
9	5.4	8.7	3.7	1.5	.09	.04	.45
10	6.3	10.3	4.6	1.5	.05	.02	.46
11	7.7	13.2	7.0	1.6	.03	.009	.77
12	6.2	6.2	4.6	1.1	.70	.68	.46
13	7.7	13.4	11.0	2.0	.17	.13	.26
14	6.9	12.7	7.2	1.5	.03	.009	.87
15	9.1	12.4	9.3	1.5	.27	.11	.93

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 22. EFFECT OF VITAMIN A STATUS ON PHYTOHEMAGLUTININ (1:500) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	--------CCPM[c] X 10^{-3}--------			
1	31.1	25.3	5.63	.48
2	43.7	41.1	7.22	.81
3	50.6	31.5	6.30	.05
4	62.8	42.3	7.96	.09
5	69.1	56.7	5.18	.11
6	43.7	32.6	7.29	.50
7	27.9	20.3	4.57	.26
8	43.4	30.5	6.45	.18
9	23.2	11.2	3.29	.02
10	17.6	13.8	2.54	.30
11	----------REPLETION----------			
12	15.6	20.8	5.79	.54
13	16.2	10.8	2.98	.24
14	17.9	12.8	3.71	.36
15	22.3	17.6	5.30	.35
16	16.9	15.3	4.59	.87
17	22.2	12.6	8.06	.42
18	18.2	17.2	5.28	.90

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 23 EFFECT OF VITAMIN A STATUS ON PHYTOHEMAGLUTININ (1:500) STIMULATED LYMPHOCYTE PROLIFERATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	-------CCPM[c] X 10^{-3}-------						
1	8.9	9.4		2.97	.92		
2	6.4	8.2		3.59	.73		
3	2.0	1.6		1.08	.78		
4	1.8	1.8		1.10	.97		
5	1.0	1.7		.68	.48		
6	2.4	1.3		.65	.25		
7	.6	1.3		.56	.39		
8	----------REPLETION----------						
9	.9	5.5	.6	1.29	.03	.007	.89
10	.6	2.3	.7	.97	.38	.17	.95
11	1.2	1.3	1.9	1.28	.90	.87	.66
12	-.2	.9	.3	.96	.70	.46	.69
13	1.9	1.6	1.8	.61	.92	.72	.84
14	1.8	4.0	2.6	.94	.28	.14	.58
15	1.6	3.8	3.5	1.66	.61	.57	.41

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells

TABLE 24. EFFECT OF VITAMIN A STATUS ON OVALBUMIN (1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

Week	Treatment: Control	Treatment: A-def	SE[a]	Prob.[b]
	----CCPM[c] X 10^{-3}---			
1	4.1	6.1	1.51	.37
2	12.2	18.2	3.39	.23
3	13.6	15.1	2.51	.67
4	18.2	18.2	2.50	.98
5	6.7	8.6	1.54	.41
6	13.2	21.7	2.17	.05
7	9.4	3.9	1.85	.05

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus the CPM of the unstimulated cells.

TABLE 25. EFFECT OF VITAMIN A STATUS ON LYSOZYME (1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	----CCPM[c] X 10^{-3}---			
4	-.2	.7	1.31	.65
5	.2	-.6	.79	.46
6	.7	.2	1.64	.82
7	2.3	-1.5	.94	.01
8	.9	0	1.00	.52
9	2.1	.3	1.41	.39
10	2.9	4.8	1.08	.28

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus the CPM of the unstimulated cells.

TABLE 26. EFFECT OF VITAMIN A STATUS ON HUMAN GAMMA GLOBULIN (1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	----CCPM[c] X 10^{-3}---			
12	.7	.6	1.92	.97
13	1.0	-.3	1.81	.65
14	-1.0	-2.1	1.04	.46
15	-.9	-.9	1.16	.99
16	.8	-.6	1.15	.58
17	-.6	-2.5	1.11	.24
18	-.6	.8	1.50	.53

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus the CPM of the unstimulated cells.

TABLE 27. EFFECT OF VITAMIN A STATUS ON OVALBUMIN (1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial II

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	----CCPM[c] X 10^{-3}---			
1	7.1	8.3	2.02	.67
2	12.1	10.5	2.88	.69
3	8.4	14.4	3.90	.30
4	9.8	8.4	2.76	.71
5	11.4	9.9	3.64	.79
6	-.1	1.7	.93	.19
7	.9	2.2	1.10	.41

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus the CPM of the unstimulated cells.

TABLE 28. EFFECT OF VITAMIN A STATUS ON HUMAN GAMMA GLOBULIN (1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	---------CCPM[c] X 10^{-3}---------						
9	-.1	2.1	-.6	.76	.05	.02	.63
10	0	4.1	-1.1	1.31	.03	.009	.56
11	-.7	3.3	.3	1.45	.15	.06	.63
12	-1.4	.2	.7	.82	.18	.57	.08
13	-.1	4.3	1.1	1.55	.16	.07	.56
14	.7	3.5	.1	1.80	.39	.18	.83
15	.3	3.0	1.2	1.15	.28	.14	.59

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 29. EFFECT OF VITAMIN A STATUS ON OVALBUMIN (.1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	----CCPM[c] X 10^{-3}---			
1	.6	3.5	.92	.04
2	2.4	4.3	1.79	.47
3	4.5	4.8	1.36	.88
4	4.7	3.6	1.95	.69
5	1.6	.4	.57	.17
6	1.9	5.3	1.54	.14
7	5.8	1.3	1.27	.03

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus the CPM of the unstimulated cells.

TABLE 30. EFFECT OF VITAMIN A STATUS ON LYSOZYME (.1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	----CCPM[c] X 10^{-3}---			
4	-3.3	-3.5	1.90	.94
5	-.2	-.7	.57	.60
6	-1.7	-2.3	1.28	.67
7	-.1	-.9	.52	.29
8	.6	.6	.72	.93
9	1.6	.3	1.14	.41
10	1.3	2.5	1.17	.43

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus the CPM of the unstimulated cells.

TABLE 31. EFFECT OF VITAMIN A STATUS ON HUMAN GAMMA GLOBULIN (.1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial I

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	----CCPM[c] X 10^{-3}---			
12	1.3	2.3	1.52	.64
13	1.4	-.7	1.66	.39
14	.2	1.5	1.04	.39
15	.6	2.1	1.41	.46
16	1.1	.9	.81	.71
17	-.6	-.8	1.76	.91
18	-1.3	.6	2.07	.54

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus the CPM of the unstimulated cells.

TABLE 32. EFFECT OF VITAMIN A STATUS ON OVALBUMIN (.1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial II

	Treatment			
Week	Control	A-def	SE[a]	Prob.[b]
	----CCPM[c] X 10^{-3}---			
1	4.5	5.5	1.77	.71
2	6.4	8.4	2.42	.57
3	4.1	7.7	2.31	.29
4	5.6	4.2	1.73	.58
5	8.3	5.8	3.04	.56
6	.6	2.2	1.15	.34
7	-.2	1.4	.66	.13

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.
[c]CPM of the stimulated cells minus the CPM of the unstimulated cells.

TABLE 33. EFFECT OF VITAMIN A STATUS ON HUMAN GAMMA GLOBULIN (.1 mg·ml^{-1}) STIMULATED LYMPHOCYTE PROLIFERATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	-------CCPM[c] X 10^{-3}------						
9	.3	.1	.5	.73	.93	.73	.84
10	-.9	2.5	-1.4	1.19	.07	.02	.78
11	-.9	1.8	-.1	1.41	.38	.19	.67
12	-1.8	0	.3	.85	.21	.50	.10
13	-.6	3.2	.7	1.17	.10	.05	.44
14	.5	1.7	.1	1.38	.72	.43	.86
15	-.2	1.1	1.3	1.03	.55	.67	.31

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.
[c]CPM of the stimulated cells minus CPM of the unstimulated cells.

TABLE 34. EFFECT OF VITAMIN A STATUS ON SERUM CORTISOL CONCENTRATIONS

Trial I

	Treatment				
Week	Control	A-def	A-rep	SE[a]	Prob.[b]
	---------ng·ml^{-1}---------				
1	10.7	9.7		1.2	.58
2	10.4	9.7		.9	.58
3	9.8	10.1		1.0	.84
4	11.3	12.1		1.2	.66
5	10.5	12.1		.9	.22
6	9.5	11.5		1.0	.29
7	10.8	10.6		.9	.87
8	10.6	10.5		.8	.93
9	12.4	11.2		1.1	.43
10	8.9	11.5		.7	.02
11	----------REPLETION----------				
12	11.3		12.5	2.0	.65
13	9.8		8.8	1.8	.69
14	12.3		8.0	1.2	.04
15	11.6		12.3	1.1	.64
16	12.1		10.4	1.4	.40
17	12.2		9.3	1.1	.11
18	12.8		9.4	1.7	.19

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 35. EFFECT OF VITAMIN A STATUS ON SERUM CORTISOL CONCENTRATIONS

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	----------ng·ml^{-1}----------						
1	2.9	3.3		.4	.55		
2	2.8	1.9		.2	.02		
3	3.1	3.1		.3	.97		
4	2.6	2.5		.2	.65		
5	2.7	2.9		.3	.65		
6	2.9	2.8		.2	.72		
7	2.9	2.9		.3	.85		
8	----------REPLETION----------						
9	3.0	3.2	2.7	.4	.64	.45	.59
10	3.1	2.7	2.9	.3	.69	.44	.71
11	3.0	2.5	3.2	.4	.56	.30	.79
12	3.1	3.7	2.5	.5	.24	.13	.42
13	3.4	2.7	3.0	.4	.36	.23	.44
14	3.9	3.6	2.5	.4	.08	.47	.03
15	3.1	2.6	2.6	.3	.39	.46	.24

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 36. EFFECT OF VITAMIN A STATUS ON PERIPHERAL BLOOD LYMPHOCYTE NUMBERS

Trial I

Week	Treatment				
	Control	A-def	A-rep	SE[a]	Prob.[b]
	-------10^6 cells per ml-----				
1	2.26	1.90		.304	.42
2	2.59	2.45		.233	.68
3	3.04	2.27		.207	.02
4	2.84	2.28		.322	.24
5	2.83	2.40		.261	.26
6	3.04	3.00		.387	.94
7	2.79	1.92		.331	.08
8	1.86	2.18		.347	.53
9	2.19	2.18		.290	.98
10	1.73	2.47		.321	.13
11	----------REPLETION----------				
12	2.22		2.49	.420	.66
13	2.26		2.47	.367	.70
14	2.44		2.50	.417	.92
15	2.80		2.08	.296	.12
16	2.20		2.23	.224	.93
17	2.21		2.22	.251	.98
18	2.36		1.85	.447	.46

[a]Standard error of the mean.
[b]Probability that a difference this large or larger could have occurred by chance.

TABLE 37. EFFECT OF VITAMIN A STATUS ON PERIPHERAL BLOOD LYMPHOCYTE NUMBERS

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	-------10^6 cells per ml------						
1	2.20	2.18		.193	.93		
2	1.89	2.04		.122	.39		
3	3.18	2.90		.226	.40		
4	2.05	1.88		.129	.37		
5	1.76	1.57		.133	.35		
6	2.77	2.50		.216	.40		
7	2.24	2.01		.282	.56		
8	----------REPLETION----------						
9	1.96	2.27	3.03	.302	.06	.55	.02
10	1.89	1.95	1.77	.263	.88	.71	.73
11	1.94	2.33	2.47	.251	.32	.69	.15
12	1.91	2.02	1.86	.325	.94	.75	.91
13	3.64	4.02	4.94	.585	.28	.72	.12
14	3.12	4.41	3.24	.454	.12	.04	.86
15	2.44	3.30	2.88	.375	.30	.19	.40

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 38. EFFECT OF VITAMIN A STATUS ON BLOOD pH

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
1	7.32	7.30		.008	.05		
2	7.33	7.31		.011	.20		
3	7.33	7.31		.011	.25		
4	7.34	7.32		.008	.13		
5	7.32	7.29		.010	.05		
6	7.34	7.31		.010	.06		
7	7.33	7.29		.013	.06		
8	----------REPLETION----------						
11	7.30	7.26	7.30	.010	.02	.006	1.00
12	7.34	7.29	7.32	.015	.08	.04	.40
13	7.34	7.30	7.33	.013	.07	.03	.59
15	7.29	7.29	7.29	.012	.94	.97	.73

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 39. EFFECT OF VITAMIN A STATUS ON BLOOD PARTIAL PRESSURE OF CARBON DIOXIDE

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmHg						
1	49.1	49.2		1.22	.92		
2	47.4	49.7		1.11	.16		
3	48.2	48.7		1.95	.80		
4	44.2	46.6		1.59	.31		
5	52.0	53.8		1.95	.54		
6	45.8	46.9		1.25	.53		
7	49.0	47.8		1.30	.55		
8	REPLETION						
11	45.7	44.6	45.5	1.31	.83	.56	.91
12	45.5	48.6	47.5	1.54	.39	.30	.38
13	41.4	45.4	42.4	1.80	.30	.14	.69
15	45.4	43.6	44.6	1.85	.79	.55	.74

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 40. EFFECT OF VITAMIN A STATUS ON BLOOD PARTIAL PRESSURE OF OXYGEN

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmHg						
1	41.9	40.4		1.11	.37		
2	45.5	43.8		1.58	.46		
3	48.2	48.7		1.52	.80		
4	50.9	46.2		1.21	.02		
5	41.4	42.9		2.39	.66		
6	43.7	41.3		1.59	.31		
7	40.4	42.4		1.48	.35		
8	REPLETION						
11							
12	40.3	37.7	41.1	1.70	.36	.17	.73
13	37.8	30.4	36.3	1.63	.02	.005	.51
15	47.5	42.8	48.2	2.60	.32	.14	.86

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 41. EFFECT OF VITAMIN A STATUS ON BLOOD SODIUM CONCENTRATIONS

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmol·L^{-1}						
1	148.9	148.4		.42	.45		
2	146.3	147.3		.56	.22		
3	147.6	147.3		.40	.56		
4	148.1	148.3		.32	.64		
5	150.2	150.5		.41	.59		
6	147.4	147.6		.23	.40		
7	148.5	148.2		.32	.59		
8	REPLETION						
11	148.5	149.3	147.6	.42	.04	.02	.15
12	149.4	150.8	149.1	.65	.17	.07	.75
13	149.2	150.5	148.6	.33	.002	.001	.17
15	157.5	159.2	157.2	1.31	.54	.28	.88

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 42. EFFECT OF VITAMIN A STATUS ON BLOOD POTASSIUM CONCENTRATIONS

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmol·L^{-1}						
1	4.8	5.0		.11	.21		
2	4.9	5.0		.11	.56		
3	4.8	5.3		.20	.20		
4	4.4	4.6		.08	.24		
5	4.9	5.0		.11	.65		
6	5.0	5.1		.14	.46		
7	5.2	5.4		.19	.36		
8	REPLETION						
11	5.1	5.5	5.3	.24	.42	.28	.47
12	5.3	5.9	5.5	.31	.44	.24	.62
13	4.7	4.9	4.8	.08	.10	.06	.26
15	5.5	5.7	5.6	.23	.89	.68	.79

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 43. EFFECT OF VITAMIN A STATUS ON BLOOD HEMOGLOBIN CONCENTRATIONS

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	g·dl^{-1}						
1	11.4	11.7		.21	.35		
2	11.1	11.6		.20	.11		
3	11.5	11.4		.19	.90		
4	10.1	10.4		.22	.25		
5	11.3	11.7		.18	.10		
6	10.8	11.2		.14	.06		
7	10.9	11.4		.19	.09		
8	REPLETION						
11	10.6	11.0	10.8	.30	.70	.43	.80
12	10.7	11.5	10.8	.38	.23	.10	.80
13	10.8	11.2	10.8	.23	.40	.18	.90
15	11.3	11.5	11.5	.24	.74	.70	.51

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 44. EFFECT OF VITAMIN A STATUS ON THE BASE EXCESS OF BLOOD EXTRACELLULAR FLUID

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmol·L^{-1}						
1	-.9	-2.5		.27	.0004		
2	-.8	-2.1		.49	.08		
3	-1.2	-2.2		.55	.19		
4	-2.2	-2.4		.44	.73		
5	.5	-1.3		.60	.06		
6	-1.4	-3.2		.57	.04		
7	-.2	-3.7		.59	.0007		
8	REPLETION						
11	-4.5	-7.4	-4.7	.71	.02	.004	.92
12	-1.5	-3.8	-1.8	.71	.08	.03	.83
13	-3.5	-4.8	-3.8	.55	.28	.13	.69
15	-5.2	-5.8	-5.1	.67	.71	.41	.92

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 45. EFFECT OF VITAMIN A STATUS ON THE BASE EXCESS OF BLOOD

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmol·L^{-1}						
1	-.2	-1.8		.23	.0002		
2	0	-1.3		.47	.08		
3	-.5	-1.5		.50	.16		
4	-1.2	-1.5		.38	.58		
5	.9	-.8		.51	.03		
6	-.6	-2.3		.52	.03		
7	.4	-2.8		.57	.001		
8	REPLETION						
11	-3.4	-6.2	-3.5	.64	.01	.003	.91
12	-.7	-2.9	-1.0	.68	.07	.02	.76
13	-2.3	-3.6	-2.6	.50	.19	.08	.64
15	-4.0	-4.6	-3.9	.59	.70	.41	.91

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 46. EFFECT OF VITAMIN A STATUS ON THE BLOOD STANDARD BICARBONATE CONCENTRATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmol·L^{-1}						
1	23.7	22.3		.20	.0001		
2	23.9	22.8		.39	.07		
3	23.6	22.6		.41	.13		
4	23.2	22.8		.31	.40		
5	24.7	23.1		.42	.02		
6	23.5	21.9		.44	.02		
7	24.2	21.5		.48	.001		
8	REPLETION						
11							
12	23.3	21.3	23.1	.58	.05	.02	.76
13	21.9	20.4	21.6	.39	.06	.02	.58
15	20.6	20.1	20.8	.44	.57	.31	.81

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 47. EFFECT OF VITAMIN A STATUS ON THE CALCULATED BLOOD BICARBONATE CONCENTRATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmol·L^{-1}						
1	24.9	23.7		.27	.006		
2	24.9	23.9		.39	.12		
3	24.6	23.8		.50	.29		
4	23.4	23.5		.45	.90		
5	26.3	25.1		.60	.18		
6	24.2	22.9		.48	.09		
7	25.4	22.6		.45	.004		
8	REPLETION						
11	21.7	19.5	21.6	.62	.03	.009	.92
12	24.0	22.6	24.1	.56	.14	.05	.94
13	22.0	21.5	21.9	.51	.77	.50	.85
15	21.3	20.5	21.2	.64	.70	.41	.94

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 48. EFFECT OF VITAMIN A STATUS ON THE BLOOD TOTAL CARBON DIOXIDE CONCENTRATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	mmol·L^{-1}						
1	26.3	25.1		.29	.01		
2	26.2	25.3		.41	.15		
3	26.0	25.2		.52	.31		
4	24.6	24.8		.49	.85		
5	27.8	26.6		.64	.21		
6	25.5	24.2		.50	.10		
7	26.8	24.0		.46	.004		
8	REPLETION						
11	23.0	20.7	22.9	.65	.03	.01	.91
12	25.3	24.0	25.4	.58	.18	.07	.88
13	23.2	22.8	23.1	.56	.87	.62	.89
15	22.5	21.8	22.5	.68	.70	.40	.95

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 49. EFFECT OF VITAMIN A STATUS ON THE BLOOD OXYGEN SATURATION

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	----------%----------						
1	65.9	61.9		2.15	.22		
2	69.4	68.1		2.43	.73		
3	70.9	67.5		1.72	.18		
4	77.7	71.8		1.44	.01		
5	70.0	64.4		2.18	.09		
6	68.9	63.6		2.62	.18		
7	63.8	64.4		2.65	.89		
8	----------REPLETION----------						
11							
12	63.7	56.8	64.1	3.28	.24	.10	.94
13	60.1	44.0	57.6	3.34	.008	.003	.59
15	70.5	65.1	71.8	3.76	.45	.22	.81

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 50. EFFECT OF VITAMIN A STATUS ON THE BLOOD OXYGEN CONTENT

Trial II

	Treatment				Probability[a]		
Wk	Control(1)	A-def(2)	A-rep(3)	SE[b]	Trt	1,3 vs 2	1 vs 3
	ml·dl^{-1}						
1	10.3	10.0		.33	.43		
2	10.5	10.9		.40	.59		
3	11.2	10.6		.26	.13		
4	10.8	10.4		.28	.33		
5	10.9	10.4		.38	.41		
6	10.3	9.8		.40	.47		
7	9.6	10.2		.39	.30		
8	REPLETION						
11							
12	9.3	9.0	9.5	.43	.68	.41	.77
13	8.9	6.7	8.5	.46	.01	.003	.58
15	10.9	10.4	11.4	.56	.45	.27	.55

[a]Probability that a difference this large or larger could have occurred by chance.
[b]Standard error of the mean.

TABLE 51. EXAMPLE ANALYSIS OF VARIANCE, HUMAN GAMMA GLOBULIN SPECIFIC SERUM IgG (TRIAL I)

Source	Degrees of Freedom	Sum of Squares	F	Pr > F
Treatment	1	5.63	1.25	.28
Block	14	141.66	2.25	.07
Error	14	62.86		
Total	29	210.16		

TABLE 52. EXAMPLE ANALYSIS OF VARIANCE, HUMAN GAMMA GLOBULIN SPECIFIC SERUM IgG (TRIAL II)

Source	Degrees of Freedom	Sum of Squares	F	Pr > F
Treatment	2	392.4	2.00	.16
Block	9	1220.7	1.38	.26
Error	18	1764.2		
Total	29	3377.4		
Contrast				
TRT 1,3 vs 2	1	372.0	3.80	.07
TRT 1 vs 3	1	20.4	.21	.65

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