VITAMIN A DEFICIENCY: SERUM CORTISOL AND IMMUNOGLOBULIN G LEVELS IN LAMBS

BY

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Chapter I

Introduction

Vitamin A, one of the earliest vitamins to be discovered, has so far 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). Many theories have been hypothesized to explain the above mentioned occurrences but the mechanisms behind these occurrences 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 and glucocorticoids. 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 correlate the relationship between vitamin A, glucocorticoids and the immune system. If one can show that vitamin A-deficient animals have increased serum glucocorticoid levels and an impaired immune system as measured by decreased serum immunoglobulin G (IgG) concentrations, one may be a step closer to understanding vitamin A's role in immunity.

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, mucous, lysozyme, tears and many others (Barrett, 1980).

Once an Ag penetrates this first line of defense it may activate a whole network of cells. Macrophages may engulf this Ag and present antigenic determinants to B and T lymphocytes. The B lympocytes (B-cell) reproduce forming plasma cells which are capable of producing immunoglobulins. The antigen-exposed T-lympocyte (T-cell) sends messages to macrophages causing them to be more actively phagocytic. The T-cell may also stimulate (helper T-cell) or suppress (suppressor T-cell) B-cell activities. Lymphokines, produced by T-cells, may attract macrophages

(chemotaxin), inhibit macrophage migration, attack foreign cells directly as well as many other functions. This overview of the immune system is simplistic and superficial at best, but one can see that the defense of the body against foreign substances is complex and 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 or humoral immune system or both affected by a vitamin A deficiency? Is this affect of the vitamin A-deficiency due directly or indirectly to the vitamin A status of the animal? 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 bacteriostatic

activity associated with lysozyme and secretory immunoglobulin A (IgA) (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 postkeratinizing 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.

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.

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.

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 splenic weight (Nauss 1979, 1985). Still others report marked decreases in relative thymus weight in rats (Bang, 1973; Krishnan, 1974; Zile, 1979; Davis, 1983). Consistantly, it has been observed that vitamin A-deficient chickens have decreased thymic and bursa of fabricius weights (Nockels, 1967). 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.

Mitogen-proliferative responses have also been assessed in thymic and splenic lymphocyte populations. Dennert and Lotan (1978) obseved no stimulatory effect of vitamin A on *in vitro* lymphocyte proliferation. Nauss (1979) observed no effect of vitamin A deficiency on concanavalin A (T-cell mitogen) responses of thymic cells. In contrast, the splenic lympocyte response to concanavalin A , phytohemaglutinin (a B and T-cell mitogen) or lipopolysaccharide (a B-cell mitogen) was significantly suppressed in vitamin A-deficient rats.

Not only does the effect of mitogen stimulation on peripheral lymphocytes seem to be effected 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.

Cell-Mediated Immunity. Cell-mediated immunity is that part of the immune system which is mediated by T-cells. T-cells contribute to a reaction known as a delayed-type hypersensitivity reaction (DTH). DTH reactions require prior sensitization by an Ag and is 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 DHT 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 mice which receive a transplant are supplemented with vitamin A (Floersheim and Bollag, 1972; Jurin and Tannock, 1972). Large doses of vitamin A cause rejection of autologous skin grafts (Boss, 1966; Floersheim and Bollag, 1972). Theoretically this should not happen because the immune system does not normally 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 it has been shown that pretreatment with vitamin A prior to tumor challenge results in a lower incidence of tumors and inhibition of tumor growth (Glaser and Lotan 1979). In a series of experiments involving syngenic tumor models (tumors from identical individuals) Dennert (1979) conclusively showed that vitamin A stimulated the induction of T-killer cells specific for the syngenic tumors studied.

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 parasites was the reason for the infections (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. 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 have been shown to 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. IgA). 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 hemaglutination 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.

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 seperately 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 causesan immediate and marked decrease in serum complement levels.

Vitamin A and Glucocorticoids. It has been observed that 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 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 has been shown to have an affect on 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.

Glucocorticoids as Immunosuppressants. The immune system is an adaptive response of the body to antigens. There are three principle cell types which 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 lympocytes 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 which is a product of B-cells. 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. It has also been observed that animals with high corticosterone blood levels showed 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 in beef calves. It was observed that this stressful period resulted in increased plasma glucocorticoid levels and a concomittant decrease in antibody titer.

Glucocorticoids exert their inhibiting powers on T-cells at primarily three levels. They inhibit interleukin 1 (IL-1) and interleukin 2 (IL-2), 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 clearence 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 occured. 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

Objectives

An experiment was conducted to examine the possible relationships among vitamin A-deficiency, cortisol and IgG levels in ewe lambs.

The specific objectives were:

1) To determine if serum cortisol levels are affected by a decrease in the vitamin A status of ewe lambs.

2) To determine if serum IgG levels vary with a decrease in the vitamin A status of ewe lambs.

3) To determine if vitamin A status influences the response of a ewe lamb to a challenge with an Ag as indicated by responses in serum IgG levels.

Chapter IV

VITAMIN A DEFICIENCY: SERUM CORTISOL AND IMMUNOGLOBULIN G LEVELS IN LAMBS

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ABSTRACT

Serum cortisol and immunoglobulin G (IgG) concentrations were measured to investigate the relationship between vitamin A status and immune function in lambs. Twenty-four crossbred ewe lambs were each fed 900 $g \cdot d^{-1}$ of a carotene-deficient diet composed of 95.5% whole oats, 3% molasses, .5% trace mineral salt and 1% limestone. All lambs were injected monthly with vitamins D and E and with selenium. The 12 control lambs also received a 100,000 IU oral dose of vitamin A palmitate in capsule form every 2 wk. All lambs were challenged by injecting them with 1 mg ovalbumin in 1 ml of Freund's complete adjuvant. At the time of

challenge, serum vitamin A levels for the control and A-deficient (A-def) lambs were 33.3 and 3.1 $ug \cdot dl^{-1}$, respectively. Blood was collected prior to and 6, 13, 20 and 34 d post-challenge. The lambs were then re-challenged using the same antigen and blood was obtained 1, 2, 6 and 22 d post-challenge. Lambs were sacrificed at the end of the second challenge period. Spleen weights were obtained and gross post-mortem observations were made at this time. Liver vitamin A concentrations were greater ($P < .0001$) in the control animals with a mean of 69.5 $ug \cdot g^{-1}$ verses 1.3 $ug \cdot g^{-1}$ for the A-def lambs. Both groups of lambs exhibited a similar growth response until day 105 at which time daily gain of the control lambs exceeded that of the A-def lambs ($P < .03$). Changes in serum IgG concentrations in response to catheterization tended to be greater in the A-def lambs. IgG response of the control lambs was greater ($P < .08$) to the first challenge and numerically greater to the second challenge. Spleen weights were greater ($P < .002$) in the A-def lambs and 11 of 12 A-def lambs contained lung abscesses as apposed to 1 of 12 for the control lambs. Results indicate that serum cortisol concentrations were unaffected by vitamin A status, but elevated serum cortisol concentrations prior to challenge may have had a

detrimental affect on the immune responsiveness in both groups of animals, throughout the study. (Key Words: Vitamin A, Cortisol, Immunoglobulin G, Immunity.)

Introduction

Numerous studies have demonstrated that both the frequency and severity of bacterial, viral and protozoal infections are increased in vitamin A-deficient (A-Def) animals (Bang and Foard, 1971; Cohen and Elin, 1974; Darip et al., 1979). It has been observed that A-Def animals have decreased splenic and thymic mass (Bang, 1973; Krishnan et al., 1976), decreased splenic lymphocyte response to mitogens (Nauss, 1979), decreased serum and secretory Ig levels (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). The physiological basis behind these observations, however, are not clearly understood.

Vitamin A has also been proposed to have an effect on glucocorticoid production. Gruber et al. (1976) observed increased plasma corticosteroid levels in A-Def rats while an elevated level of glucocorticoids was also suggested by Webb et al. (1969) as the reason for polyuria in A-Def sheep. Perek and Kendler (1969) noted that a vitamin A-deficiency impaired the growth of the bursa of Fabricius. They concluded that this impaired growth was due to an

increased concentration of adrenal steroids which are known to have lympholytic activity. Vitamin A deficiency is also associated with protein energy malnutrition which has been shown to cause elevated levels of plasma glucocorticoids in children (Leonard and MacWilliam, 1964). Glucocorticoids have a wide range of effects on virtually every phase and component of the immune system (Cupps and Fauci, 1982). Glucocorticoids decrease serum immunoglobulins (Ig) levels (Butler and Rossen, 1973), decrease proliferation of T lymphocytes, decrease production of immune interferon (Munck et al., 1984), and decrease monocyte chemotaxis as well as phagocytosis (Rinehart, 1974). Any circumstance which may cause an elevated level of glucocorticoids could have a detrimental effect on the ability of an animal to respond to an antigen (Ag).

The objective of this study was to investigate the relationships among vitamin A deficiency, serum cortisol concentrations and immune function in lambs. Serum cortisol and IgG concentrations were measured through the depletion period and in response to antigenic challenge.

Procedure

Twenty-four crossbred ewe lambs weighing approximately 10 kg were blocked according to weight and randomly assigned to either a control or A-Def treatment. The lambs were treated for internal parasites[1] (Levasole) and vaccinated against clostridium[2] (Ultrabac-7). The lambs were housed under constant lighting conditions on a raised expanded metal pen 2.5m x 10.0m equipped with automatic nipple waterers and fed twice daily. The lambs were fed as a group at the rate of 900g·head^{-1}·d^{-1} of a whole oat diet (table 1) and injected monthly with vitamins D[3] (50,000 IU·mo^{-1}) and E[4] (600 IU· mo^{-1}) and with selenium[5] (.05 mg·kg^{-1}·mo^{-1}). The control lambs also received 100,000 IU oral doses of vitamin A palmitate[6] in capsule form every 2 wk. Eight weeks into the feeding period, a bleeding regimen was initiated in which the animals were bled (50 ml) via jugular puncture and weighed every 2 wk for 4 mo. Throughout the study, blood samples were consistantly taken

[1]Pitman-Moore, Washington Crossing, NJ. 08560.
[2]Beecham Laboritories, Bristol, TN. 37620.
[3]Taylor Pharmacal Co., Decatur, IL. 62525.
[4]Anchor Laboritories, St. Joseph, MO. 64502.
[5]Burns-Biotic, Omaha, NE. 68103.
[6]R.P. Scherer Corp., Clearwater, FL. 33518.

TABLE 1. DIET COMPOSITION[a]

Item	%
Oats (IFN 4-03-309)	95.5
Molasses (IFN 4-04-696)	3.0
Limestone (IFN 6-02-632)	1.0
Trace mineral salt	.5
Vit D[b]	+
Vit E[c]	+
Selenium[d]	+
Vit A[e]	+/-

[a]As fed basis.

[b]Individual mo injection of 50,000 USP.

[c]Individual mo injection of 600 IU.

[d]Individual mo injection of .05mg·kg^{-1}.

[e]100,000 IU vit A palmitate in capsule form every 2 wk for control lambs only.

at 0900 h. Serum was harvested and frozen (-20 C) for later analysis of vitamin A, IgG and cortisol concentrations.

Upon completion of the 4-mo bleeding period, jugular catheters were placed into each ewe lamb and the animals were placed into metabolism stalls to facilitate easier accessibility to the catheters. Blood samples (10 ml) were taken 4, 5, 7, 9, 16 and 43 d post catheterization with serum samples again being frozen for latter analysis. The lambs were then challenged with 1 mg ovalbumin[7] in 1 ml of Freund's complete adjuvant[7]. Half of this solution was injected intramuscularly in the hind leg with the remainder of the solution injected subcutaneously in five different sites along the animals rib area. Blood was obtained prior to and 6, 13, 20 and 34 d post-challenge. Upon completion of the first challenge period the lambs were rechallenged as before with blood taken 1, 2, 6 and 22 days post-challenge. At the end of the second challenge period the lambs were weighed, sacrificed and spleen and kidney weights obtained. Liver samples were taken at this time and frozen (-20 C) for later analysis of liver vitamin A concentrations. Gross post-mortem observations were also made at this time.

[7]Miles Laboritories Inc., Naperville, IL. 60566.

Serum vitamin A, cortisol and nonspecific IgG levels were monitored throughout the study. 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). Nonspecific serum IgG concentrations were elucidated by a modification of the radial immunodiffusion technique (Mancini et al., 1965) and serum cortisol concentrations were determined using a single antibody radioimmunoassay.[8]

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

Results and Discussion

Serum vitamin A concentrations at the beginning of the bleeding regimen were 42.3 and 22.3 $ug \cdot dl^{-1}$ for the control and A-def lambs, respectively. Vitamin A concentrations for the control lambs remained relatively constant due to vitamin A supplementation whereas vitamin A concentrations for the A-def lambs were reduced to 3.5 $ug \cdot dl^{-1}$ just prior to catheterization and 3.1 $ug \cdot dl^{-1}$ at the time of the first ovalbumin challenge (figure 1). Vitamin A deficiency symptoms included nervousness, muscular weakness, staggered gait, convulsions and death. The muscular weakness and staggered gait may have been the result of an increase in cerebrospinal fluid pressure which is known to occurr in vitamin A deficient states (Eaton et al. 1961). Liver vitamin A concentrations observed upon completion of the study were 69.5 and 1.3 $ug \cdot g^{-1}$ for the control and A-def lambs, respectively.

Control and A-def lambs exhibited a similar growth response until d 105 at which time weight ($P < .12$) and daily gain ($P < .03$) of the control lambs exceeded that of the A-def lambs. Weights of the A-def lambs dropped from 47.6 kg on d 105 to 36.3 kg on d 207. Control lambs gained more during this same period, 49.6 kg to 51.8 kg (figure

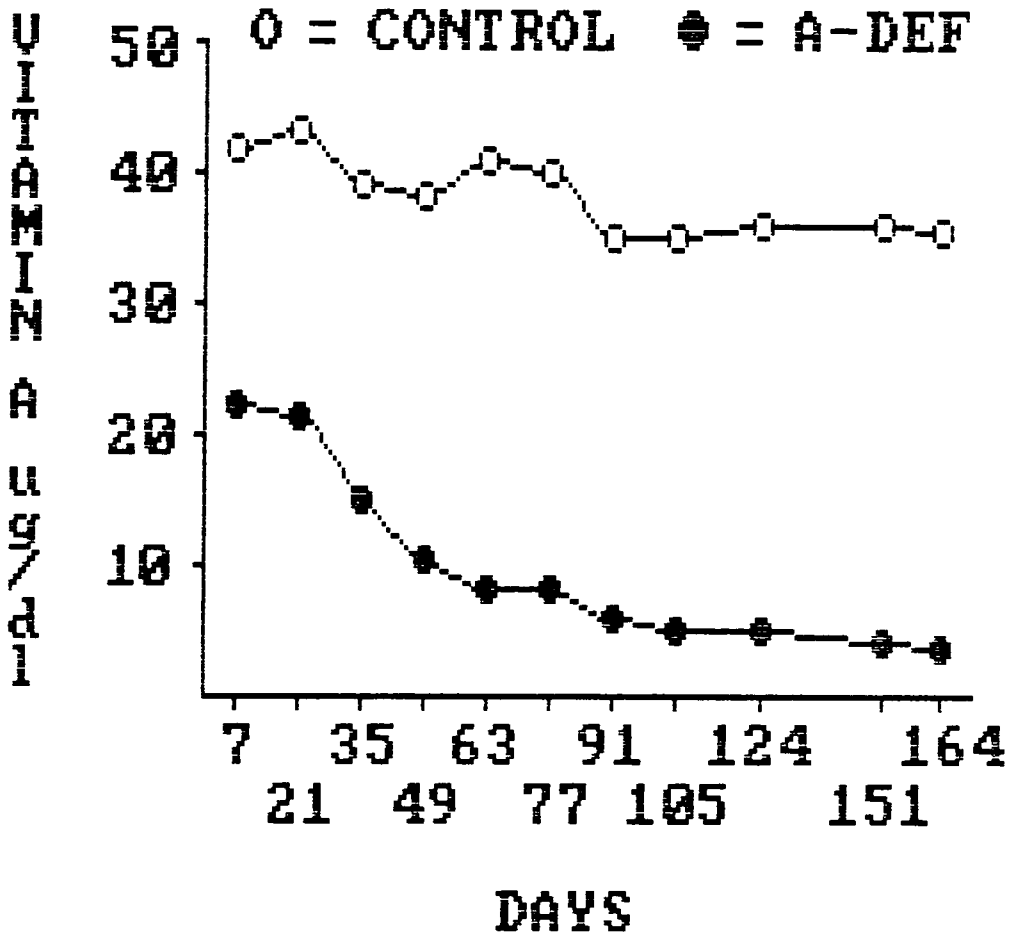

FIGURE 1. Mean serum vitamin A concentration in control and A-def lambs.

2). The weight loss observed in the A-def lambs from d 105 to d 207 is likely the result of a loss of appetite which is observed in vitamin A deficiencies (Anzano et al. 1979). Differences in intake were not measurable, however, since the animals were fed as a group.

Serum cortisol concentrations prior to catheterization followed an unexplained 56 d cyclic pattern with the A-def lambs tending to have the lower mean concentration (figure 3). Serum cortisol concentrations were lower in the A-def lambs on d 92 ($P < .10$) and 105 ($P < .02$). Following catheterization (d 108), cortisol concentrations in the control lambs continued to decline, whereas cortisol concentrations in the A-def lambs rose resulting in a numerically greater concentration in the A-def lambs. Just prior to the first ovalbumin challenge (d 105), serum cortisol concentrations were greater ($P < .04$) in the A-def lambs but declined by d 157 (figure 4). The cortisol concentrations in the control lambs responded inversely to the A-def lambs with an increase in cortisol concentration from days 151 to 157. Serum cortisol concentrations increased in both groups of lambs up to the second ovalbumin challenge (d 185). Serum cortisol concentrations were not significantly different prior to

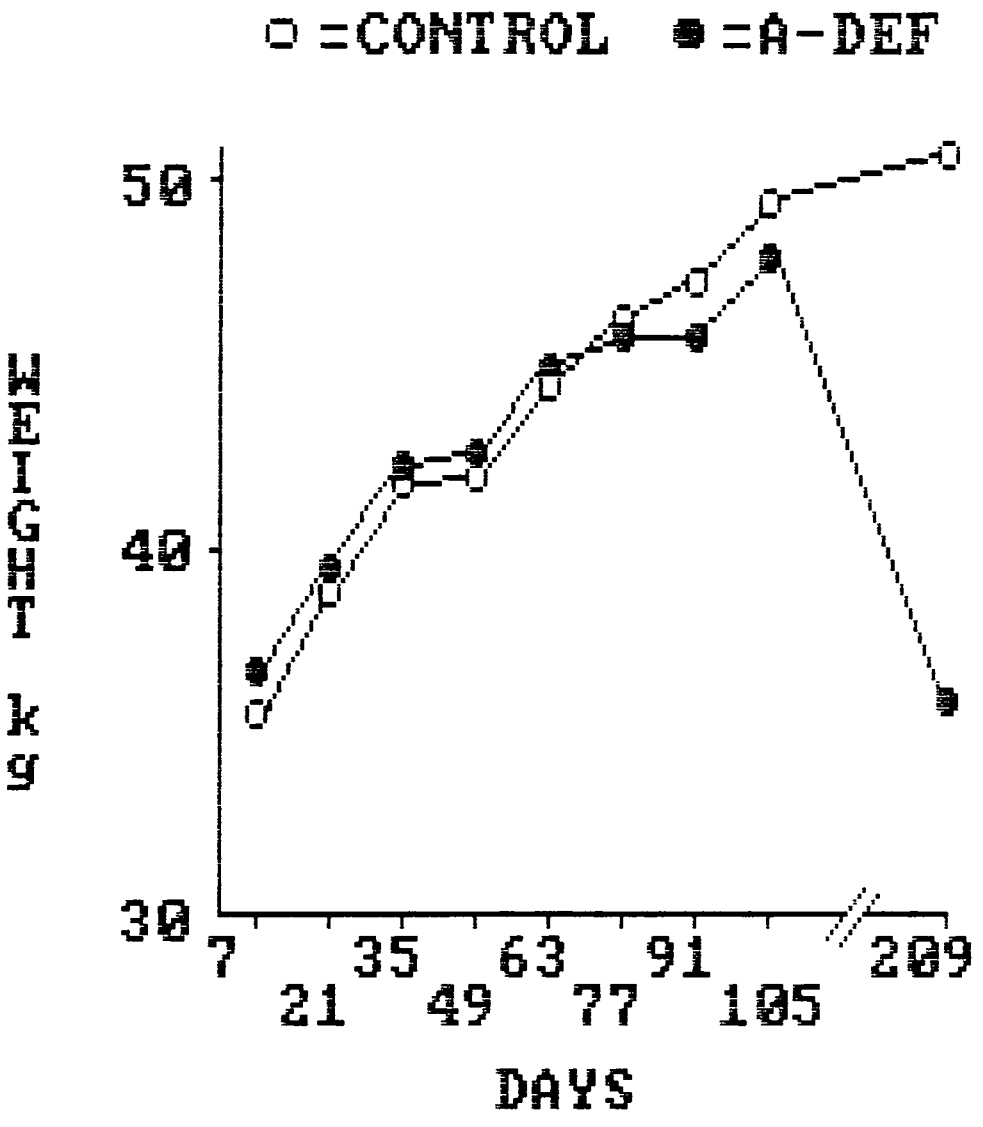

FIGURE 2. Weights of control and A-def lambs throughout the study.

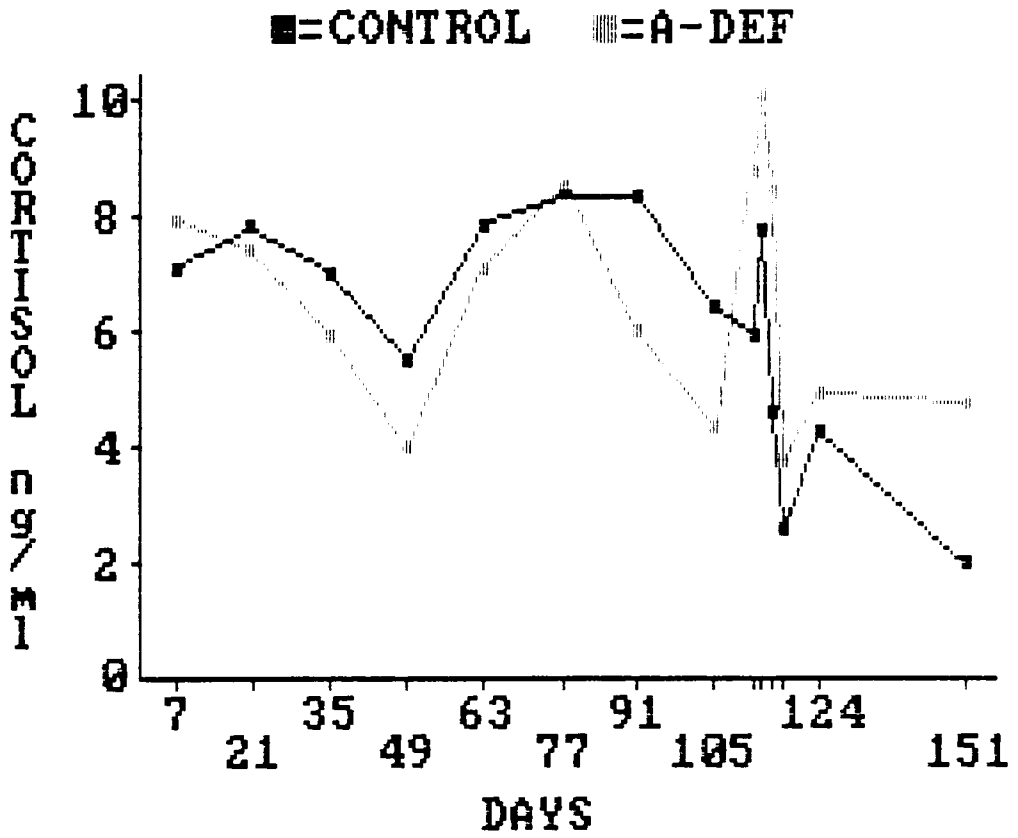

FIGURE 3. Mean serum cortisol concentrations. Lambs were catheterized on day 108.

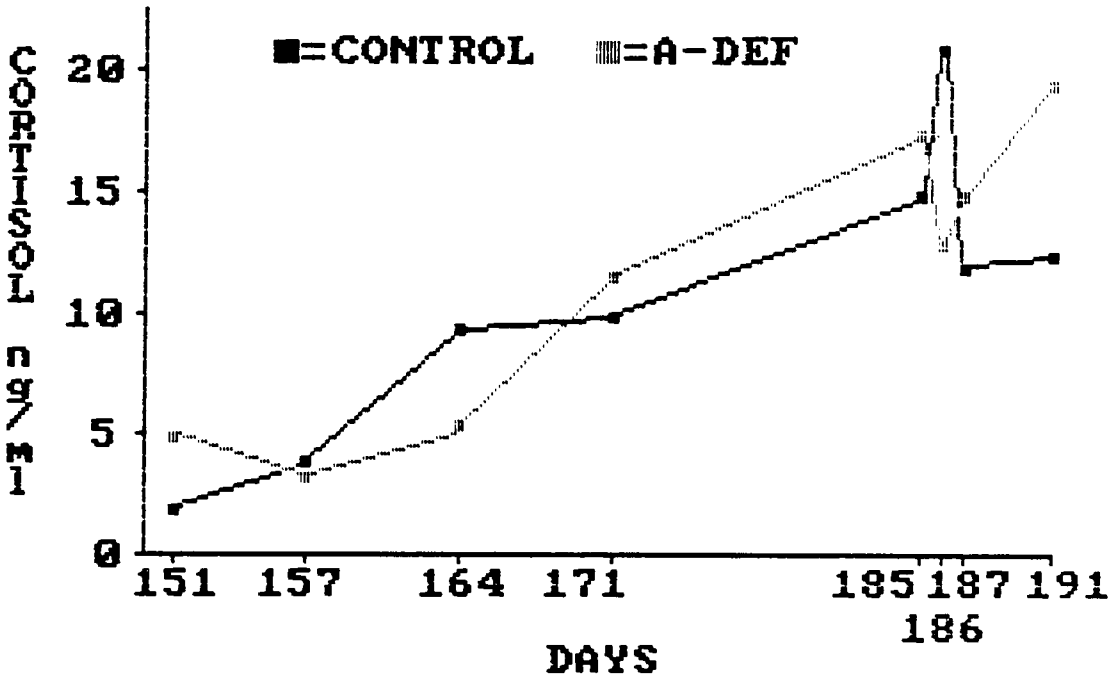

FIGURE 4. Mean serum cortisol concentrations. Lambs were challenged on days 157 and 185.

the second ovalbumin challenge but the A-def lambs tended to have a higher numerical value. Serum cortisol concentrations obtained from the first bleeding following the second ovalbumin challenge showed a decrease in concentrations in the A-def lambs and an increase in the control lambs. Cortisol concentrations increased throughout the remainder of the study in the A-def lambs but decreased in the control lambs. Vitamin A status seemed to have no consistent affects on serum cortisol concentrations.

Looking closely at the serum cortisol concentrations during the catheterization period as well as during both challenge periods, it was observed that animals with higher cortisol concentrations prior to challenge did not respond with an increase in cortisol as did the animals with the lower initial concentration. These observations point to the negative feedback of increased blood cortisol concentrations inhibiting the secretions of adrenalcorticotropic hormone and consequently reducing cortisol secretions (Swenson, 1984).

IgG concentrations were significantly higher in the A-def lambs on d 105 as compared to the control lambs (figure 5). Prior to catheterization, serum IgG concentrations were higher ($P < .002$) in the A-def lambs

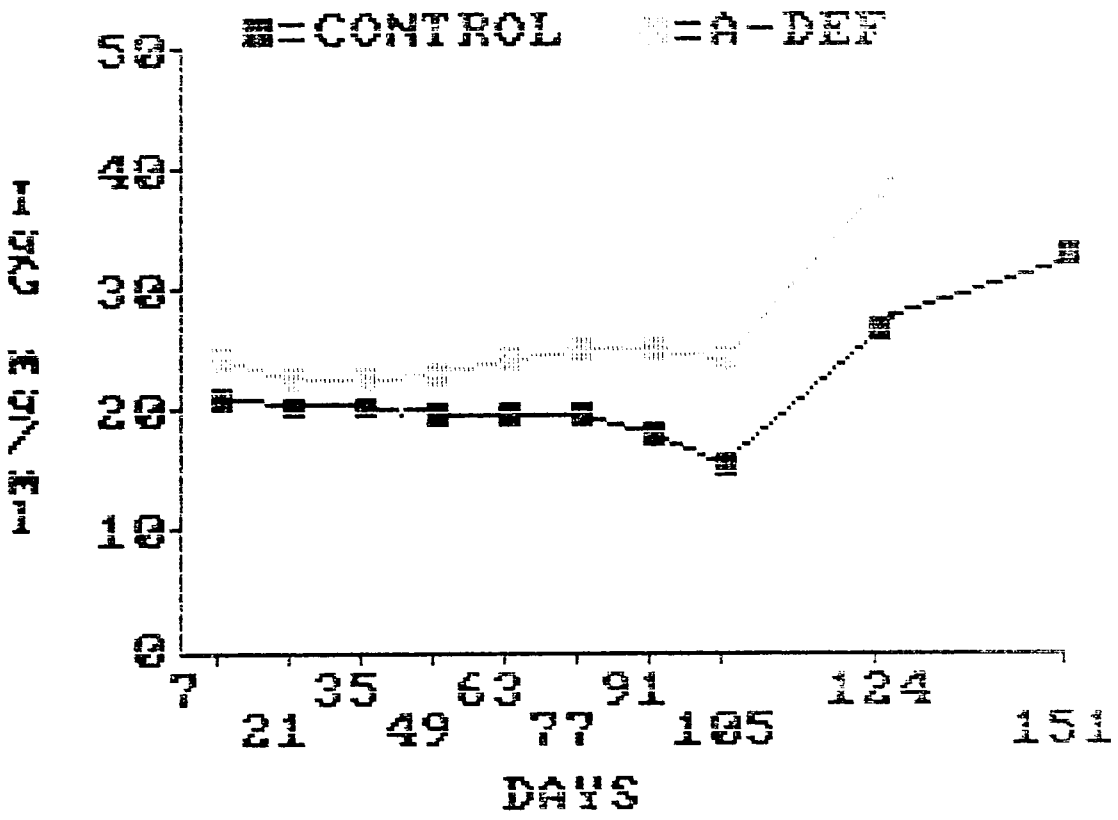

FIGURE 5. Mean serum IgG concentration in control and vitamin A deficient lambs. Lambs were catheterized on day 108.

with means of 24.2 $mg \cdot ml^{-1}$ versus 15.5 $mg \cdot ml^{-1}$ for the control lambs. Both groups of lambs responded with a linear ($P < .0001$) increase in serum IgG concentrations following catheterization. Treatment affects were not significantly different but the A-def lambs responded with a greater numerical increase in IgG. IgG concentrations in A-def lambs rose from 24.2 $mg \cdot ml^{-1}$ on d 105 to 48.11 $mg \cdot ml^{-1}$ on d 151 for an increase of 23.9 $mg \cdot ml^{-1}$. IgG concentrations in control lambs increased from 15.6 $mg \cdot ml^{-1}$ to 32.9 $mg \cdot ml^{-1}$ for an increase of 17.3 $mg \cdot ml^{-1}$ over this same period. On the day of the first ovalbumin challenge (d 151), serum IgG concentrations were larger ($P < 03$) in the A-def lambs (figure 6). Both control and A-def lambs responded to the challenge with a linear ($P < .0001$) increase in IgG concentrations. However, the A-def lambs did not respond with as great an increase in IgG concentrations as did the controls as indicated by the time by treatment interaction ($P < .0001$) which was observed during this time period. IgG concentrations just prior to the second ovalbumin challenge were not significantly different between treatments.

A characteristic drop in IgG concentrations was observed in both groups of animals 2 d following the second challenge. This observed drop in IgG concentrations is due

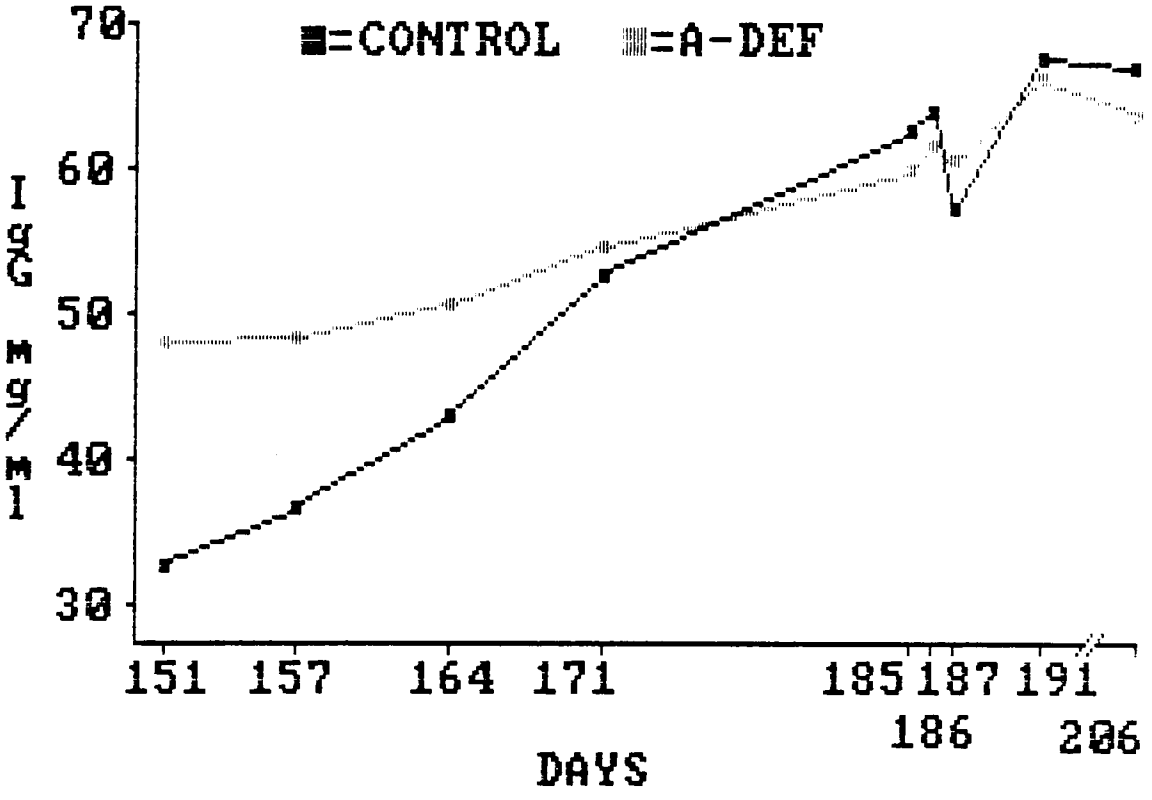

FIGURE 6. Mean serum IgG concentrations. Lambs were challenged on days 151 and 185.

to the IgG complexing with the newly injected antigen (Barrett, 1983). A numerically greater decrease in IgG concentrations was observed in the control lambs indicating a possible increase in the proportion of ovalbumin specific antibodies in these lambs. Since the control lambs responded to the first ovalbumin challenge with a greater increase in IgG, it would seem likely that they would contain a higher proportion of ovalbumin specific antibodies. Following this characteristic drop in IgG concentrations, both groups of lambs increased their serum IgG concentrations in response to the second ovalbumin challenge. The increase was not significantly different among treatments but the control lambs had a greater numerical increase than the A-def lambs. Control lambs increased their IgG concentrations 10.3 $mg \cdot ml^{-1}$ from d 187 to d 191 while the A-def lambs increased their IgG concentrations 5.9 $mg \cdot ml^{-1}$ over this same period. Immunoglobulin G concentrations on d 206 were not significantly different among treatments.

Graphing the cumulative change in IgG concentrations for the first and second challenge period demonstrates more clearly the responses of the control and A-def lambs to the ovalbumin challenges (figure 7). Both groups of lambs

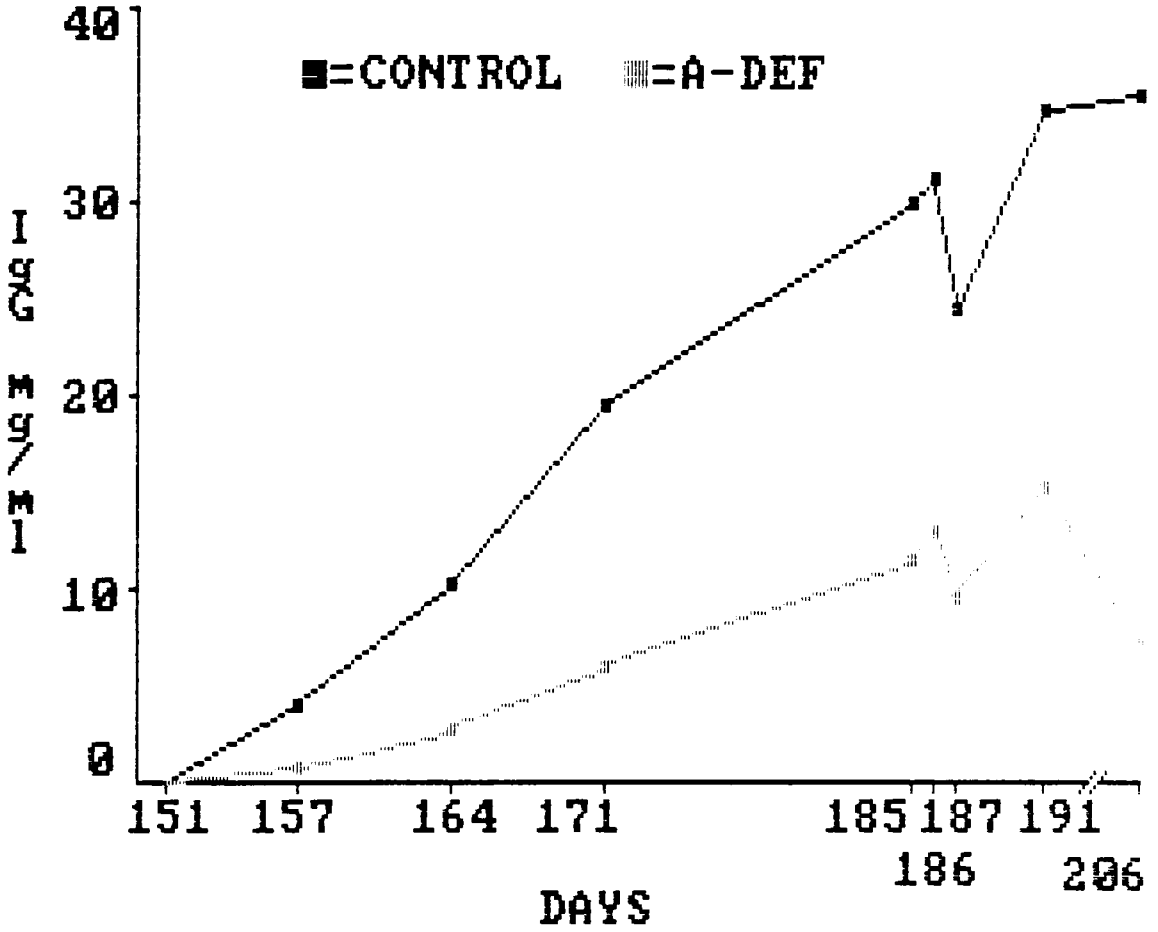

FIGURE 7. Cumulative change in IgG concentration for the two ovalbumin challenge periods. Lambs were challenged on days 151 and 185.

responded linearly to the first challenge ($P < .0001$) with the control lambs responding with a greater increase in IgG ($P < .03$). Both the decrease in IgG concentrations from d 186 to d 187 and the increase in IgG concentrations from d 187 to d 191 though not significantly different, were larger for the control lambs.

If one looks closely at each challenge period, including catheterization, one can see that on each occassion, the group of animals with the highest cortisol concentration prior to challenge did not respond with as great an increase in IgG as the group with the lower cortisol concentration. Prior to catheterization the control lambs had greater serum cortisol concentrations ($P < .002$) and did not respond with as great a numerical increase in IgG as the A-def lambs. Prior to the first ovalbumin challenge the A-def lambs had a greater serum cortisol concentration ($P < .04$) and did not respond as well to the challenge as the control lambs ($P < .08$). Finally, prior to the second challenge, the A-def lambs again had elevated cortisol concentrations over the control lambs and again did not respond with as great a numerical increase in IgG. This is similar to the findings of Gwazdauskas et al. (1978) who observed that weaned beef

calves with elevated levels of cortisol prior to challenge did not respond with as great an increase in IgG as those calves with lower cortisol concentrations. Cortisol is a known immunosuppressant which can detrimentally affect Ig production (Butler and Rossen, 1973), macrophage function (Rinehart et al., 1974) and T-cell function (Munk et al., 1984) to name a few. Another possible reason for the A-def lambs not responding as well to the first ovalbumin challenge may involve their initial IgG concentration. The A-def lambs had a greater initial IgG concentration than the control lambs ($P < .03$) and IgG concentrations in both groups of lambs at the end of the first challenge period were similar. The differences in immune responsiveness measured by the slopes of these response lines therefore may be due more to the starting point rather than a true estimate of immune responsiveness.

Spleen weight was also observed to be significantly larger ($P < .002$) in the A-def lambs (table 2). This is in contrast to Nauss et al., (1985) who observed no differences in spleen weights in control and A-def rats. Bang, (1973) and Krishnan et al. (1976) in further contrast reported decreased splenic weights in A-def animals. The enlarged spleens in the A-def lambs may have been in

TABLE 2. SPLEEN WEIGHTS OF CONTROL AND VITAMIN A-DEFICIENT LAMBS

Treatment	Weight[a], g	SE[b]
Control	81.5	3.5
A-deficient	128.7	15.4

[a]Means differ ($P < .002$).

[b]Standard error of mean.

response to infections which are more prominent in A-def animals. It was also observed that 11 of 12 A-def lambs had lung abscesses whereas this number was only 1 of 12 for the control lambs. Whether these lung abscesses were due to vitamin A's affects on epithelial tissue, the immune system or a combination of the two is not known.

Results of the present study indicate that serum cortisol concentrations were unaffected by vitamin A status but that serum IgG concentrations increased as vitamin A status decreased. Changes in IgG concentrations in response to antigenic challenge were variable among treatments but elevated serum cortisol concentrations prior to antigenic challenge may of had detrimental affects on the immune responsiveness in both groups of animals, throughout the three challenge periods.

Chapter V

General Discussion

It has been observed in this study that the group of lambs with elevated serum cortisol concentrations prior to catheterization and the ovalbumin challenges, did not respond with as great an increase in IgG as did those lambs with the lower serum cortisol concentration. This observation was not restricted to a particular treatment but was observed in both groups of lambs depending on the challenge period. The affect of cortisol on IgG concentrations in response to the three challenges are more likely a result of decreased production as apposed to increased catabolism. Butler and Rossen (1973), observed both an increase in catabolism and a decrease in production of Ig in humans treated with corticosteroids. They concluded, however, that the decreased serum Ig concentrations were primarily due to a decrease in Ig production.

In general, glucocorticoids exert their greatest affects on Ig production when they are in high concentrations just prior to antigenic challenge. The glucocorticoids appear to modulate early events in the immune response as opposed to significantly affecting

established responses (Cupps and Fauci, 1982). Observations in the present study seem to agree with the above generalization. In all three challenge periods, the group of animals with the greatest increase in serum IgG concentrations always had a lower serum cortisol concentration just prior to antigenic challenge. The serum cortisol concentration, however, did not remain lower than the other group throughout the entire challenge period.

The area of research dealing with nutritional affects on the immune system is a complex area involving numerous factors. As mentioned previously, vitamin A is necessary for many physiological functions. A study utilizing vitamin A-deficient animals, therefore, may have several confounding factors which may mask the real cause and affect. Many parameters used to measure immune responsiveness also leave some doubt as to the real cause and affect. Serum Ig production for example, can be monitered as was done in this study, but because of the complex network of cells and soluble factors which interact to produce an Ig response, measuring the final product tells one nothing about what is happening in the intermediate steps. One confounding factor associated with vitamin A may be vitamin A's affects on thyroid hormones.

Conflicting observations have been reported involving vitamin A's affects on thyroid function. By measuring basal energy metabolism as well as serum thyroid hormone concentrations, numerous investigators have concluded that vitamin A deficiency results in hypothyroidism (Jaya Rao and Khan, 1974; Oba and Kimura, 1979; Morley et al., 1981 and Nockels et al., 1984). Others report an increase in serum thyroid hormone concentrations as vitamin A status decreases (Morley et al., 1978 and Garcin and Higueret, 1980). Typical symptoms associated with hyperthyroidism include polyuria, polyphagia, weight loss, weakness and fatigue as well as nervousness. All of these symptoms have been observed either in the present study or in others associated with vitamin A-deficient animals. Increased serum thyroid hormone concentrations have also been implicated in causing increased spleen weights (VanWyck et al., 1976) which were also observed in this study. The increase in spleen weights may have also been caused by an increase in workload associated with a decrease in liver function and/or an increased infection rate. The affects of vitamin A deficiency on thyroid function are still somewhat ambiguous, but one must not overlook the possible

implications when interpreting data associated with vitamin A-deficient animals.

Vitamin A has also been found to be linked to protein metabolism as well. It is important to remember when interpreting data involving vitamin A-deficient people in the developing countries that vitamin A deficiencies are usually associated with protein energy malnutrition (PEM). PEM may represent a major confounding factor in many of these investigations.

In laboratory animal experiments where conditions can be much more rigidly controlled, protein metabolism may still pose a problem. Growth depression is one of the characteristic symptoms of vitamin A deficiency, especially in the young growing animal. Body weight gain of deficient rats is depressed early in the course of the deficiency even before noticeable changes in food intake occur (Orr and Richards, 1934). This inefficient weight gain appears to be related to a decreased utilization of dietary protein (Brown and Morgan, 1948). These observations tend to negate the concept that appetite loss and reduced feed intake are responsible for the decreased weight gain , although they do become complicating factors once the deficiency becomes more advanced. Madjid et al. (1978) demonstrated that the

observed decrease in serum complement levels thought to be associated with vitamin A deficiency was instead associated with a protein deficiency. Krishnan et al. (1974) also observed that the response to sheep red blood cells was diminished to the same extent in both vitamin A-deficient rats and pair-fed controls. It would appear that general undernutrition and/or a decrease in dietary protein utilization have a major influence on the immune responsiveness of an animal.

Recently, dietary protein type and plasma amino acid profiles have been observed to have an affect on B-cell and T-cell immune responses in mice (Bounous and Kongshavn, 1985a,b and Wannemacher, 1980). Since vitamin A deficiencies have an affect on dietary protein utilization, the decrease in immune responsiveness observed in vitamin A-deficient animals may in some degree be due to a change in quantity and/or distribution of plasma amino acids.

One of the interesting observations noted in this study dealt with the incidences of lung abscesses. As reported earlier, 11 of 12 A-def lambs had lung abscesses whereas the incidence was only 1 of 12 in the control lambs. The cause or causes of these lung abscesses may be many. Increased susceptibility to infections is due in part

to vitamin A's role in maintaining the functional integrity of epithelial and mucosal surfaces. A decrease in integrity of these anatomical barriers increases the virulence of infectious agents. Vitamin A may also affect the number and/or activity of pulmonary macrophages. It has been observed by many investigators (Cohen and Elin, 1974; Hof and Emmerling, 1979 and Rhoades and Oliver, 1980) that vitamin A has an affect on macrophage numbers and activity. The conflicting results, however, generate more questions than answers. Secretory IgA (sIgA) antibodies are also involved in mucosal immunity and therefore may have had an affect on the incidence of lung abscesses. Sirisinha et al. (1980) observed a decrease in sIgA in the intestinal fluid of A-def rats. It has also been reported that sIgA levels in the nasal washings of children with PEM were decreased especially in those complicated with vitamin A deficiencies (Sirisinha et al., 1975).

The above discussions demonstrate one of the major problems encountered in biological research and that is the problem of confounding factors. The wide range of physiological changes associated with a vitamin A deficiency as well as the complexity of the immune system may overshadow the real cause and affect. Researchers in

the area of nutrition and immunity must face these problems and deal with them as best they can in their experimental designs.

Chapter VI

Summary

Serum cortisol and immunoglobulin G (IgG) concentrations were measured to investigate the relationship between vitamin A status and immune function in lambs. Twenty-four crossbred ewe lambs were each fed 900 $g \cdot d^{-1}$ of a carotene-deficient diet composed of 95.5% whole oats, 3% molasses, .5% trace mineral salt and 1% limestone. All lambs were injected monthly with vitamins D and E and with selenium. The 12 control lambs also received a 100,000 IU oral dose of vitamin A palmitate in capsule form every 2 wk. All lambs were challenged by injecting them with 1 mg ovalbumin in 1 ml of Freund's complete adjuvant. At the time of challenge, serum vitamin A levels for the control and A-deficient (A-def) lambs were 33.3 and 3.1 $ug \cdot dl^{-1}$, respectively. Blood was collected prior to and 6, 13, 20 and 34 d post-challenge. The lambs were then re-challenged using the same antigen and blood was obtained 1, 2, 6 and 22 d post-challenge. Lambs were sacrificed at the end of the second challenge period. Spleen weights were obtained and gross post-mortem observations were made at this time.

Liver vitamin A concentrations were greater ($P < .0001$) in the control animals with a mean of 69.5 $ug \cdot g^{-1}$ verses 1.3 $ug \cdot g^{-1}$ for the A-def lambs. Both groups of lambs exhibited a similar growth response until day 105 at which time daily gain of the control lambs exceeded that of the A-def lambs ($P < .03$). Changes in serum IgG concentrations in response to catheterization tended to be greater in the A-def lambs. IgG response of the control lambs was greater ($P < .08$) to the first challenge and numerically greater to the second challenge. Spleen weights were greater ($P < .002$) in the A-def lambs and 11 of 12 A-def lambs contained lung abscesses as apposed to 1 of 12 for the control lambs. Results indicate that serum cortisol concentrations were unaffected by vitamin A status but elevated serum cortisol concentrations prior to challenge may have had a detrimental affect on the immune responsiveness in both groups of animals, throughout the study.

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APPENDIX A

Catheter Preparations and Procedures

Catheter Production. The jugular catheters consisted of medical grade Silastic[1] tubing (.076 cm ID x .165 cm OD) and were approximately 45 cm in length. Care was taken to round the intravascular tips so as to reduce surface irregularities that could possibly cause damage to the vein resulting in increased fibrosis and catheter malfunction. The rounding of the intravascular tips was performed using a Dremel Moto-tool[2] equiped with a fine sandpaper bit. The catheter was washed in a sonic bath with a non-ionic detergent solution, rinsed with deionized water and autoclaved.

Catheter Insertion. The catheters were inserted into the vein via a 13 gauge needle. Approximately 15 cm of the tubing was inserted into the vein with the remainder being fed back to the middle of the animal's shoulders. The catheter was sutured to the skin at the point of entry and also on the back between the shoulder blades. This was performed by doubling a 4 cm long piece of cloth tape

[1]Dow Corning Corp., Midland, MI. 48640.
[2]Dremel MFG. Racine, WI. 53406.

around the tube so that the tube was located in the center. The tape was then sutured to the skin.

The exterior end of the catheter was placed in xylene and allowed to soak for 15 s. Xylene causes the tip to swell increasing the ease with which the 18 gauge leur stub adapter[3] can be inserted. A stopcock was placed into the leur stub adapter and the catheter was flushed with approximately 5 ml of a heparinized (40 units·ml^{-1}) .9% sterile saline solution. The necks of the lambs were then loosely wrapped with an elastic, adhesive bandage[4] followed by a wrapping of cloth tape.

Maintainence of the catheters involved flushing with a heparinized (40 units·ml^{-1}), .9% sterile saline solution (5 ml) after each blood withdrawl. If blood was not withdrawn the catheters were flushed twice weekly.

[3]Clay Adams, Parsippany, NJ. 07054.
[4]Johnson and Johnson, New Brunswick, NJ. 08903.

APPENDIX B

Serum Vitamin A Analysis

Seperation. 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

[1]Bausch and Lamb, Spectronic 20.

transmittance read at 616 mu within 5 s from the time the trifluoroacetic acid was added.

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 C

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 D

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.

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

The liquid was decanted in this way with the tubes being 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 read.

Total count tubes were also read 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 E

IgG Determination

Preparation of Standards. Standards were prepared by adding 4 mg of sheep IgG[1] to 1 ml of phosphate buffered saline[2] (PBS). This mixture was then serially diluted to obtain concentrations of 4.0, 2.0, 1.0, .50, .25 and .125 mg/ml IgG.

Gel Plate Preparation. Three agarose-immunodiffusion tablets[3] were added to 14 ml of a solution containing .5% w/v polyethylene glycol 4000 in 16 X 125 mm culture tubes. The solution was refluxed in a boiling water bath until the tablets were completely dissolved. The tubes were then placed in a 56 C water bath until the solution had cooled to this temperature (10 min). An aliquot of this solution (11.5 ml) was pipetted into another 16 X 125 mm tube containing 250 ul affinity purified rabbit anti-sheep IgG, Fc fragment[1] and 570 ul PBS. The mixture was immediatly vortexed and poured onto a plate of glass (10 cm X 9 cm). The glass plate was set atop a leveling table[3] and kept warm by a hot plate that had been placed underneath the

[1]Pel-Freez Biologicals, Rogers, AR. 72756.
[2].02 M Sodium Phosphate, .25 M NaCl, pH 7.6.
[3]Bio-Rad Laboritories, Richmond, CA. 94804.

leveling table. After the agarose solution had been poured onto the plate, the burner was removed and the gel allowed to solidify.

Inoculation of Gel Plate. Wells were punched into the agar utilizing a plastic template and tubular cutter. A vacuum was then applied to this same tubular cutter to remove the agar plugs from the wells. To these wells was added 1 ul of a standard or serum sample. The serum samples were first diluted 20 fold with PBS before they were pipetted into the wells. Pipetting samples into the wells was performed using a capillary tube pipettor[4]. After the wells were filled with 1 ul of solution, they were placed in a humidifying chamber and incubated for 48 h at 4 C. The humidifying chamber consisted of a desiccator with the desiccant being replaced with water. Opaque precipitan rings formed around the wells after 48 h. The plates were placed on a colony counter[5] and the diameter of the rings was measured using a calibrated ruler[4].

[4]Helena Labs, Beaumont, TX. 77704.
[5]American Optical Corp., Buffalo, NY. 14215.

Concentrations of the standards were plotted versus the opaque ring diameter^2 and the unknowns were determined from this curve by linear regression.

APPENDIX F

TABLES

TABLE 3. SERUM VITAMIN A CONCENTRATIONS IN CONTROL AND VITAMIN A-DEFICIENT LAMBS

	Treatment			
Days[a]	Control	SE[b]	A-def	SE[b]
	----------ug/dl----------			
7	42.5	2.1	22.3	2.4
21	43.2	1.7	20.8	2.0
35	38.4	2.0	14.2	1.6
49	38.3	1.9	10.5	1.4
63	40.8	1.6	7.8	1.1
77	39.3	2.1	7.2	1.0
91	33.0	1.4	5.1	.7
105	33.1	1.3	3.5	.6
124	33.3	1.2	3.4	.6
151	33.3	1.2	3.1	.5
164	33.2	1.2	3.0	.5

[a]Means for control and A-def differ ($P < .01$) for all dates.

[b]Standard error of mean.

TABLE 4. WEIGHTS OF CONTROL AND VITAMIN A-DEFICIENT LAMBS THROUGHOUT THE STUDY

	Treatment			
Days	Control	SE[a]	A-def	SE[a]
	kg			
7	35.4	1.8	36.7	2.0
21	38.9	1.9	39.5	2.0
35	42.0	2.0	42.3	2.2
49	42.1	1.9	42.6	2.2
63	44.5	1.9	45.0	2.3
77	46.3	2.0	45.8	2.6
91	47.5	1.9	46.0	2.8
105	49.8	2.1	48.0	3.1
209[b]	50.8	1.3	36.2	3.8

[a]Standard error of mean.

[b]Means for control differ from A-def ($P < .05$).

TABLE 5. CORTISOL CONCENTRATIONS IN CONTROL AND A-DEFICIENT LAMBS PRIOR TO THE FIRST OVALBUMIN CHALLENGE

	Treatment			
Days	Control	SE[a]	A-def	SE[a]
	----------ng/ml----------			
7	7.2	.9	8.0	1.1
21	7.9	1.4	7.4	1.2
35	7.1	1.3	6.0	.8
49	5.8	1.1	4.2	.6
63	7.9	1.5	7.2	.9
77	8.5	1.4	8.6	.9
91	8.5	1.8	6.1	.7
105[b]	7.1	1.1	4.4	.4
112	6.0	1.4	8.8	2.9
113	7.8	1.7	10.2	3.4
115	4.7	.7	8.5	2.9
117	2.7	.6	3.8	.7
124	4.3	2.8	5.0	.8
151[b]	2.1	.3	4.8	.9

[a]Standard error of mean.

[b]Controls differ from A-def ($P < .05$).

TABLE 6. SERUM CORTISOL CONCENTRATION IN CONTROL AND A-DEFICIENT LAMBS THROUGH THE TWO OVALBUMIN CHALLENGE PERIODS

	Treatment			
Days	Control	SE[a]	A-def	SE[a]
	----------ng/ml----------			
151[b]	2.1	.3	4.8	.9
157	4.1	1.1	3.5	.5
164	9.7	3.8	5.6	2.0
171[c]	10.0	4.5	11.7	2.7
185	15.1	3.3	17.5	3.6
186	21.0	4.0	13.2	2.7
187	12.6	3.2	15.2	3.2
191	12.5	3.8	19.5	5.5

[a]Standard error of mean.

[b]Controls differ from A-def ($P < .05$).

[c]Controls differ from A-def ($P < .10$).

TABLE 7. SERUM IGG CONCENTRATIONS IN CONTROL AND A-DEFICIENT LAMBS PRIOR TO THE FIRST OVALBUMIN CHALLENGE

	Treatment			
Days	Control	SE[a]	A-def	SE[a]
	mg/ml			
7	21.0	2.0	24.2	1.7
21	21.0	1.9	22.4	1.5
35	20.8	1.6	22.3	1.2
49[c]	19.3	1.7	23.1	1.2
63[c]	19.5	1.3	24.0	1.7
77[c]	19.6	1.3	24.8	2.0
91[b]	17.8	1.1	25.1	1.8
105[b]	15.6	.9	24.2	1.7
124[c]	26.6	4.9	38.6	4.5
151[d]	33.0	4.1	48.1	4.2

[a]Standard error of mean.

[b]Controls differ from A-def ($P < .01$).

[c]Controls differ from A-def ($P < .05$).

[d]Controls differ from A-def ($P < .10$).

TABLE 8. SERUM IGG CONCENTRATIONS IN CONTROL AND A-DEFICIENT LAMBS THROUGH THE TWO OVALBUMIN CHALLENGE PERIODS

	Treatment			
Days	Control	SE[a]	A-def	SE[a]
	mg/ml			
151[b]	33.0	4.1	48.1	4.2
157	37.3	4.2	48.4	4.1
164	43.4	4.2	51.0	3.8
171	52.8	3.8	54.9	3.6
185	63.2	4.0	60.7	3.5
186	64.4	4.3	62.3	3.3
187	57.8	3.4	60.9	2.8
191	68.1	5.1	66.8	3.4
206	67.7	4.3	64.2	4.7

[a]Standard error of mean.

[b]Controls differ from A-def ($P < .10$).

TABLE 9. CUMULATIVE IGG CHANGE IN CONTROL AND A-DEFICIENT LAMBS FOR THE TWO OVALBUMIN CHALLENGE PERIODS

	Treatment			
Days	Control	SE[a]	A-def	SE[a]
	mg/ml			
151	0	-	0	-
157[c]	4.3	.9	.3	1.3
164[b]	10.5	2.0	2.9	1.9
171[b]	19.8	3.4	6.2	2.1
185[c]	30.2	5.2	11.7	2.7
186[d]	31.4	5.7	13.2	3.5
187	24.8	4.4	9.7	5.1
191	35.1	6.4	15.6	5.6
206	35.7	8.0	7.6	7.3

[a]Standard error of mean.

[b]Controls differ from A-def ($P < .01$).

[c]Controls differ from A-def ($P < .05$).

[d]Controls differ from A-def ($P < .10$).

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VITAMIN A DEFICIENCY: SERUM CORTISOL AND IMMUNOGLOBULIN G LEVELS IN LAMBS

by

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(ABSTRACT)

Serum cortisol and immunoglobulin G (IgG) concentrations were measured to investigate the relationship between vitamin A status and immune function in lambs. At the time of challenge, serum vitamin A levels for the control and A-deficient (A-def) lambs were 33.3 and 3.1 $ug \cdot dl^{-1}$, respectively. Blood was collected prior to and 6, 13, 20 and 34 d post-challenge. The lambs were then re-challenged using the same antigen and blood was obtained 1, 2, 6 and 22 d post-challenge. Lambs were sacrificed at the end of the second challenge period. Spleen weights were obtained and gross post-mortem observations were made at this time. Liver vitamin A concentrations were greater ($P < .0001$) in the control animals with a mean of 69.5 $ug \cdot g^{-1}$ verses 1.3 $ug \cdot g^{-1}$ for the A-def lambs. Both groups of lambs exhibited a similar growth response until day 105 at which time daily gain of the control lambs exceeded that of the A-def lambs ($P < .03$). Changes in serum IgG concentrations

in response to catheterization tended to be greater in the A-def lambs. IgG response of the control lambs was greater ($P < .08$) to the first challenge and numerically greater to the second challenge. Spleen weights were greater ($P < .002$) in the A-def lambs and 11 of 12 A-def lambs contained lung abscesses as apposed to 1 of 12 for the control lambs. Results indicate that serum cortisol concentrations were unaffected by vitamin A status but elevated serum cortisol concentrations prior to challenge may have had a detrimental affect on the immune responsiveness in both groups of animals, throughout the study.