PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **182**, 393–398 (1986)

The Influence of Supplemental Vitamins A and E on Ovine Humoral Immune Response[1] (42357)

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Abstract. These experiments determined if supplemental vitamins A and/or E would enhance ovine antibody responses. All-rac-α-tocopheryl acetate was fed to lambs approximately 6 months old (30 to 40 kg) at levels of 33 (controls), 121, 276, 396, and 476 mg/kg of feed (which are total vitamin E levels). Primary and secondary immunizations with 10 mg keyhole limpet hemocyanin (KLH) were given. A nonlinear dose response of serum antibody titers was observed and the 476 mg vitamin E/kg treatment significantly enhanced ($P < 0.05$) the peak primary response over controls. Retinyl acetate fed at five levels ranging from 7000 (the control level) to 97,400 IU/kg feed failed to influence antibody production to 10 mg KLH of lambs about 6 months old (29 to 41 kg). There was no detectable response to an ovalbumin antigen (100 mg). Neonatal lambs were injected with retinyl palmitate or the carrier of the injected vitamin. These lambs failed to raise antibody titers to either of the antigens administered (10 mg KLH, 100 mg ovalbumin). This was apparently due to a neonatal period of immune paralysis to certain antigens. A preliminary study showed that no KLH-specific antibodies are detectable in lambs immunized earlier than 7 weeks of age. Lambs in this age range were utilized in the last trial in which four treatments were applied: 3000 mg oral vitamin E, 400,000 IU injected vitamin A, 4 ml of the injectable vitamin A carrier, or no treatment. Half of the animals in each of these groups were immunized with 15 mg KLH and 1 ml *Brucella ovis* bacterin and the other half served as nonimmunized controls. No significant differences in titers to KLH were observed. Lambs receiving 3000 mg vitamin E or the carrier produced secondary peak anti-*B. ovis* titers higher ($P < 0.05$) than those of the untreated controls.

Both injection and feeding of high levels of vitamin A have resulted in elevated antibody production to certain antigens in mice and chickens (1–7). Feeding high levels of vitamin E to mice, rats, guinea pigs, chickens, swine, and sheep has also been found to increase antibody titers (4, 5, 8–13). Immunity enhancement by means of dietary supplementation or injection of a vitamin would be both a practical and economical means of lowering disease-related losses in livestock production. It could be particularly beneficial to neonatal ruminants, which rely upon passively obtained colostral antibodies for much of their immune protection. Stimulation of endogenous antibody production in this age group with its high incidence of disease could prove very beneficial. Therefore, the following experiments were conducted to determine if supplementation of vitamins A or E to sheep would enhance their humoral immunity. The effects of several routes of vitamin administration on ovine antibody responses to two protein and one killed pathogen antigen were studied. The factor of animal age at immunization was also examined.

Materials and Methods. *Experimental animals.* All lambs were housed in outdoor pens with open shed shelter and provided with feed and water *ad libitum.* The lambs were given *Clostridium perfringens* type C and D toxoids, ovine contagious ecthyma vaccine, and Fenarsenate paste wormer. Weight gains and feed intakes of the older lambs in the first two trials were monitored every 2 weeks. These animals were fed a basal diet of dehydrated alfalfa pellets which were ground, the appropriate amount of vitamin A or E mixed in, and repelleted. The younger lambs in the last two trials remained with their dams until weaned at approximately 3 months of age. In all trials, animal age or weight, sex, and breed were equalized among treatments.

[1] Supported in part by the Agricultural Experiment Station, Colorado State University. Published as Scientific Series Paper No. 3020.

0037-9727/86 $1.50

The keyhole limpet hemocyanin (Calbiochem, La Jolla, Calif.) and ovalbumin (Sigma Chemical Company, St. Louis, Mo.) antigens used in these experiments are both soluble proteins which were dissolved in phosphate-buffered saline (PBS; pH = 7.2), then precipitated with 13.3% aluminum hydroxide. The nonimmunized lambs in Experiment 4 received PBS with 13.3% added aluminum hydroxide. *B. ovis* bacterin (Colorado Serum Co., Denver, Colo.), a cellular antigen containing 10^9 killed *B. ovis* cells/ml, was alum-adsorbed. All antigens were administered subcutaneously.

In each experiment the sheep were bled weekly. Blood was drawn from the jugular vein into nonheparinized vacutainer tubes. Samples were allowed to clot at room temperature, the clot was rimmed, and the tube centrifuged for 10 min. The serum was drawn off into sterile tubes and stored frozen (0°C) until analyzed.

Experiment 1. The ability of high dietary vitamin E to enhance the primary and secondary antibody responses of crossbred lambs (Suffolk × Hampshire) of mixed sexes was tested. Nine lambs were assigned to each of the five treatment rations: all-rac-α-tocopheryl acetate (Rovimix E-50%) at levels of 33, 121, 276, 396, and 476 mg per kg of diet (these are total levels, basal diet plus supplemental vitamin E as analyzed by Hoffmann–La Roche, Inc., Nutley, N.J.). On Day 1 the sheep were bled. Three weeks later they were bled again and immunized with 10 mg KLH (5 mg/ml PBS). The sheep were bled six more times at 7-day intervals. Twenty-one days after the first immunization, KLH was readministered in the same dosage to test for the secondary immune response.

Experiment 2. This experiment was designed to parallel Experiment 1, substituting vitamin A for vitamin E. Forty-five commercial lambs about 6 months old (mixed sex) with initial body weights of 29 to 41 kg were assigned nine each to the five treatment rations: 7000 (control level added to meet requirement), 37,000, 53,800, 68,300, and 97,400 IU retinyl acetate (Rovimix A-650) per kilogram of feed (as analyzed by Hoffmann–La Roche). Two weeks after being allotted to the treatments, the lambs were bled and immunized with 10 mg KLH (5 mg/ml PBS) and 100 mg ovalbumin (50 mg/ml PBS). Blood was drawn weekly for the next 6 weeks. Twenty-one days after the first antigen administration the sheep were reimmunized with the same doses of KLH and ovalbumin.

Experiment 3. The effects of injectable vitamin A and the carrier substance used in the injectable vitamin were tested for their ability to enhance the antibody response of neonatal lambs. Forty lambs (Suffolk and Hampshire of mixed sex), ranging in age from 11 to 28 days at immunization, were allotted 10 each to the four treatment groups: single intramuscular (im) injections of 1 ml vitamin A carrier,[2] or 50,000, 100,000, or 200,000 IU retinyl palmitate in this carrier. One week later, all lambs were bled and immunized with 10 mg KLH (5 mg/ml PBS) and 100 mg ovalbumin (50 mg/ml PBS), followed by weekly bleedings for the next 3 weeks. Reimmunization was not performed.

Experiment 4. A preliminary trial was conducted to determine the minimum age at which KLH administration would produce a detectable antibody response in lambs. Based on the results, Experiment 4 utilized lambs 47 to 67 days old and 9 each were assigned to eight treatments. Due to the wide range of birth dates of the lambs it was necessary to conduct the experiment in two shifts. Forty-two lambs were started on trial 2 weeks after the first 30 older lambs. Since the split was made by age, lambs from all eight treatments were represented in each of the two shifts. The eight treatments were divided into four immunized and four nonimmunized groups. The four treatments in each of these divisions were no vitamins given, oral (encapsulated) vitamin E (Rovimix E-50%), im injected vitamin A (BASF-Wyandotte, Germany), and im injected vitamin A carrier (BASF-Wyandotte, Germany).[3] There were 3 days of vitamin administration. On the first, four capsules of vi-

[2] Contents of this carrier stated by supplier (Hoffmann–La Roche, Inc., Nutley, N.J.) as 7.2 g glycerin, 20.0 g emulphor, 0.01 g thimerosol, 0.01 g disodium edetate, 0.13 g butylated hydroxyanisole (BHA), 0.13 g butylated hydroxytoluene (BHT); the pH was adjusted to 4.0 and made to 100 ml with distilled water.

[3] Contents of this carrier stated by the supplier (BASF–Wyandotte, Germany) as caprylic/caproic esters of propylene glycol, BHA, BHT, ethanol, benzyl alcohol, TWEEN-80, mono- and diglycerides of oleic acid, and emulphor EL 620.

tamin E (1000 mg), 2 ml vitamin A (200,000 IU), or 2 ml of the carrier were given to the vitamin E-, vitamin A-, and carrier-treated animals, respectively. Four days later, the same groups received four capsules vitamin E (1000 mg), 1 ml vitamin A (100,000 IU), or 1 ml of carrier. This exact pattern was repeated 3 days later, so that the untreated groups received no supplemental vitamins, the vitamin E groups received 3000 mg all-rac-α-tocopheryl acetate, the vitamin A groups received 400,000 IU retinyl propionate, and the carrier groups received 4 ml of that substance. Four days after the last day of vitamin administration all animals were bled and those in the immunized groups given 15 mg KLH (5 mg/ml PBS) and 1 ml of *B. ovis* bacterin at separate sites. The nonimmunized lambs received 2 ml of PBS. Blood was drawn weekly for the next 6 weeks. All animals were again challenged with the same dosage 21 days after the first immunization.

Antibody titer analyses. Antibodies against KLH in Experiments 1 through 3 and against ovalbumin in Experiments 2 and 3 were measured by microtiter passive hemagglutination (PHA). The method (14) employed a modification of sheep red blood cell (SRBC) formalinization: to fresh, washed SRBC was added a 25% volume of 40% formalin in dialysis bags. This was agitated at 4°C for 3 hr, the dialysis bags were broken, and cold agitation was continued for 12 to 18 hr. The PHA procedure was adapted to microtiter plates and results reported as the $\log_2$ of the inverse of the last serum dilution to show a positive PHA pattern.

In Experiment 4, anti-KLH and anti-*B. ovis* titers were determined by indirect ELISA (enzyme-linked immunosorbent assay) (15). Polyvinyl microtiter plates were coated with 0.05 μg KLH or 0.10 μg of a *B. ovis* cell suspension for these tests. Absorbances were read in a Dynatech Micro-ELISA reader. They were then adjusted to the mean positive control and divided by the high-end figure of a 99% confidence interval around the absorbances of negative control sera. Results are reported as this ratio of sample absorbance to negative control absorbance.

Statistical analyses. The data from the above experiments were subjected to analysis of variance. When positive F tests were obtained, differences among the means were examined by Duncan's new multiple-range test (16).

Results and Discussion. Antibody responses of sheep about 6 months old fed high levels of vitamin E were examined in Experiment 1. There were no significant differences in weight gains or feed intake among treatments. Animals fed 476 mg all-rac-α-tocopheryl acetate per kilogram of feed produced significantly higher ($P < 0.05$) titers to KLH (second bleeding after primary immunization) than did those fed 33, 121, or 396 mg/kg feed (Fig. 1). The effect that dietary vitamin E was greater in enhancing the primary rather than the secondary response has previously been reported in mice (17). The nonlinear dose response seen here has been demonstrated in other species given high levels of dietary vitamin E (8, 18, 19) and indicates a possible effect of the vitamin on the reticuloendothelial system (20).

In Experiment 2, lambs about 6 months old were fed five levels of vitamin A and primary and secondary antibody titers to KLH and ovalbumin were determined. No significant

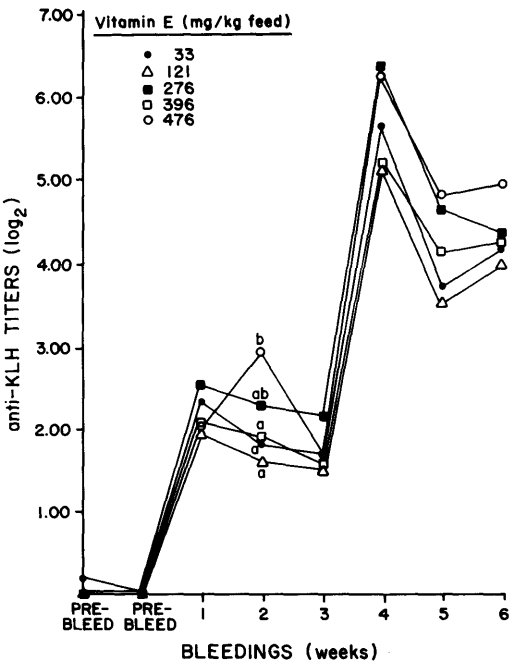

FIG. 1. Anti-KLH titers (PHA) of lambs fed all-rac-α-tocopheryl acetate (Experiment 1). Primary immunization at second prebleed; secondary immunization at bleeding 3 (3 and 6 weeks, respectively, after start on experimental diets). Letters signify statistical difference ($P < 0.05$).

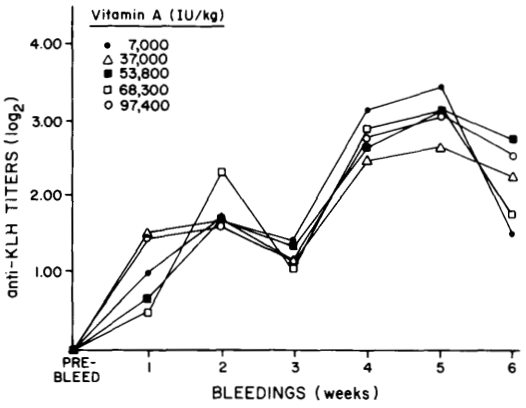

FIG. 2. Anti-KLH titers (PHA) of lambs fed retinyl acetate (Experiment 2). Primary immunization given at prebleed; secondary immunization at bleeding 3 (two and six weeks, respectively, after start on experimental diets).

differences in animal weight gains or feed intakes were observed. Forty-four percent of the lambs did not respond by producing detectable titers to ovalbumin and these data were not analyzed for treatment effects. There were no significant differences in anti-KLH titers among the treatments (Fig. 2).

During Experiment 3, lambs 4 to 21 days old were injected with either vitamin A carrier or 50,000, 100,000, or 200,000 IU vitamin A and, 1 week later, immunized with KLH and ovalbumin. Within one-half hour of antigen administration all animals became prostrated with increased respiration. Though this was perhaps due to the stress of crowding and handling such young lambs, no secondary immunization was performed for fear of causing anaphylactic shock. Forty-three percent of the animals did not respond serologically to KLH and 81% did not respond to ovalbumin. The data were therefore not analyzed for treatment effects. It was unexpected that these neonatal lambs did not respond to KLH, an antigen against which they are immunocompetent as a fetus (21) and at about 6 months old (Experiments 1 and 2). The cause for this lapse is perhaps a combination of nonspecific antibody suppression by maternal antibodies, as shown in calves (22, 23) and the lingering effects of immunosuppressive corticosteroids produced by lambs at parturition (24, 25). This theory is further supported by the preliminary trial conducted prior to Experiment 4, which showed that lambs needed to be 7 weeks old at immunization for detectable anti-KLH titers to be produced. Repetition of this trial utilizing normal and colostrum-deprived lambs with concomitant measurement of serum corticosteroids might provide valuable information about the immunocompetence of neonatal lambs and the advisability of the widespread practice of implementing immunization regimens in lambs only a few days old. These results are also supported by another author who determined that lambs 70–100 days old produce a significantly lower antibody response to chicken red blood cells than do lambs 7 to 8 months of age (26). The reasons given are similar to reasons suggested by us, but as yet no conclusive data are available to substantiate these theories.

In a preliminary study conducted prior to Experiment 4, eight Rambouillet lambs that were 35 to 42 days old at antigen administration failed to develop detectable primary titers to KLH while seven lambs that were 42 to 52 days old did. In Experiment 4, lambs 47 to 67 days old were treated with oral vitamin E, in-

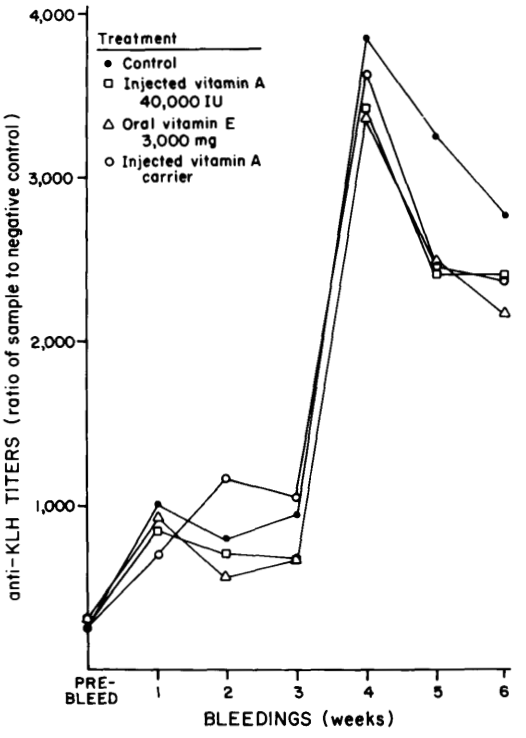

FIG. 3. Anti-KLH titers (ELISA) of lambs 47 to 67 days old when immunized (Experiment 4). Primary immunization at prebleed; secondary immunization at bleeding 3.

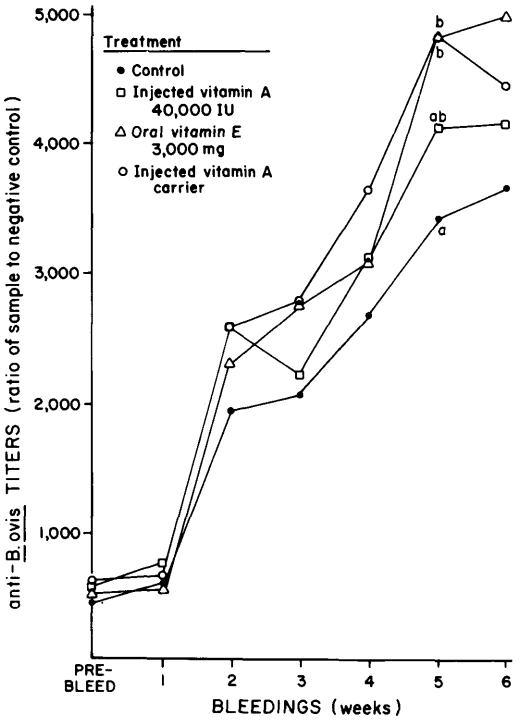

FIG. 4. Anti-*B. ovis* titers (ELISA) of lambs 47 to 67 days old when immunized (Experiment 4). Primary immunization at prebleed; secondary immunization at bleeding 3. Letters signify statistical difference ($P < 0.05$).

jected vitamin A, injected vitamin A carrier, or nothing at all. Half of the lambs in each of these treatments were immunized with KLH and *B. ovis* and half received PBS. The primary and secondary antibody response patterns to KLH (Fig. 3) were well delineated, though the primary responses were low. No significant differences in anti-KLH titers were observed among treatments. Antibody titers of the nonimmunized animals remained at or below those of the negative control sera. The *B. ovis* ELISA test revealed that all immunized animals responded well to the antigen (nonimmunized animals had slightly elevated background titers: <1.5 times negative control sera). Significant differences in titers ($P < 0.05$) were seen at the sixth bleeding (secondary response) in sheep receiving injected carrier and oral vitamin E relative to the controls (Fig. 4). The titers of the vitamin A-injected animals were nonsignificantly elevated above those of the no-vitamin controls.

The lack of effect of oral vitamin E supplementation on KLH antibody responses was probably due to the amount given (3000 mg over an 8-day period); the 476 mg vitamin E/kg feed dose which caused elevated anti-KLH titers in Experiment 1 is calculated to have provided those lambs with 27,400 mg vitamin E per day for 63 days. Bolus feeding of vitamin E resulted in enhanced secondary response antibody production to *B. ovis*. Surprisingly, the vitamin A carrier substance was also found to behave as an adjuvant in the secondary anti-*B. ovis* response; both vitamin E and the carrier possess antioxidant properties which could possibly have been responsible for the enhanced responses.

It is apparent from this research that dietary supplementation of vitamin E at very high levels may be beneficial to the humoral immune response of sheep, whereas bolus administration of the vitamin for a short time before immunization may be sufficient for some antigens but not for others. Vitamin A, however, whether injected or fed, does not seem to possess the same adjuvant effect in sheep that has been found with its use in other species. Perhaps the most important insight gained from these experiments is that neonatal lambs may not be serologically capable of meeting the challenge presented to their developing immune system by the immunization schedules routinely used in the sheep production industry.

The authors would like to thank Hoffmann-La Roche, Inc. (Nutley, N.J.) for supplying Rovimix E-50%, Rovimix A-650, and all ingredients for the preparation of injectable retinyl palmitate and carrier. Thanks also to BASF-Wyandotte (Germany) for providing injectable retinyl propionate and carrier.

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Received July 12, 1985. P.S.E.B.M. 1986, Vol. 182.
Accepted April 18, 1986.