Effect of Supplemental Vitamin E on the Immune System of Calves\textsuperscript{1,2}

P. G. REDDY, J. L. MORRILL, H. C. MINOCHA, M. B. MORRILL, A. D. DAYTON, and R. A. FREY

Departments of Animal Sciences and Industry, Laboratory Medicine, Statistics, and Anatomy and Physiology
Kansas State University
Manhattan 66506

ABSTRACT

The effect of vitamin E on immune responses of Holstein calves was investigated. Treatments were: 0, 1400, and 2800 mg of dl-\(\alpha\)-tocopheryl acetate given orally at weekly intervals or 1400 mg of dl-\(\alpha\)-tocopherol weekly by injection. Calves were fed milk for 6 wk and then fed a complete calf starter ad libitum. Calves were on experiment until they were 12 wk of age.

Lymphocyte stimulation indices were significantly higher for calves given the high amount of oral supplementation and for injected calves than for unsupplemented calves. There were no significant differences at any of the individual weeks between unsupplemented and orally supplemented calves. Injected calves showed significantly higher values than unsupplemented calves at wk 4 and than all other calves at wk 8. There were no significant differences in the concentrations of immunoglobulins G\textsubscript{1} and G\textsubscript{2} among treatments. Immunoglobulin M was significantly higher at wk 6 in calves given the high amount of oral supplementation than in all other calves. At wk 12, serum from calves given the high amount of oral supplementation and calves given injections inhibited infectious bovine rhinotracheitis viral replication in tissue cultures as compared with those of unsupplemented calves.

In supplemental experiments serum \(\alpha\)-tocopherol and lymphocyte stimulation indices of yearling heifers determined 7 d after a single injection of 2000 IU of dl-\(\alpha\)-tocopherol were significantly higher than preinjection values. In vitro addition of vitamin E to lymphocyte cultures did not increase phytohemagglutinin-induced blastogenesis.

INTRODUCTION

Nutrition plays a significant role in the development and function of the immune system (1, 28). The level of particular nutrients may affect not only humoral and cell-mediated immune responses but also several nonspecific humoral factors such as C-reactive protein, lysozymes, \(\beta\)-lysins, and hormones (9), which regulate the immune responses.

Continued high morbidity and mortality of calves warrant research emphasis on optimizing the immune response and necessitate improving the understanding of the role of nutrition. Very few attempts have been made to study the effect of single nutrients on the immune response of calves. Stern et al. (32) demonstrated an immunostimulatory effect of supplemental vitamin A in calves from birth to 5 wk. Supplemenal vitamin C maintained higher concentrations of immunoglobulin G (IgG) in plasma in calves deprived of colostrum as compared with calves deprived of both colostrum and vitamin C (3). Another nutrient that has a beneficial effect upon the immune system is vitamin E. Several research papers demonstrating the stimulatory effect of vitamin E on the humoral immune response of chicks, mice, turkeys, and pigs have been reviewed (23, 33). Recently vitamin E also has been shown to regulate the cell-mediated immune response of dogs (21, 29), pigs (22), mice (7, 8, 35), and rats (2). Cipriano et al. (6) observed a trend toward higher cell-mediated immune response in dairy calves given 1 g of supplemental vitamin E/d. The very high amount of vitamin E used in this study may have

\textsuperscript{1} Contribution 85-255-J, Kansas Agricultural Experiment Station, Manhattan 66506.

\textsuperscript{2} Supported in part by Hoffmann-La Roche, Nutley, NJ 07110.
partially masked the enhancing effect on the immune response. The optimum supplemental vitamin E to observe a clear immunopotentiation effect was probably less than the one used in the study (6).

Bovine respiratory diseases continue to be a serious problem and infectious bovine rhinotracheitis (IBR) virus is one of the principal causes (14). Morbidity due to respiratory diseases appears to be high among weaned calves shifted from hutches to group pens. Vitamin E may affect several humoral factors, for example, serum corticosterone (35) and prostaglandins (18) and thus influence replication of pathogens within the host.

Objectives of Experiment 1 were to study the effect of supplemental vitamin E on the immune system of dairy calves from birth to 3 mo under normal herd management conditions and to study the effect of serum from supplemented calves on the replication of IBR virus in tissue cultures. Experiment 2 was intended to study the effect of supplemental vitamin E on cell-mediated immune response of yearling heifers and Experiment 3 to study the effect of in vitro addition of vitamin E on lymphocyte blastogenesis.

MATERIALS AND METHODS

Experiment 1: Experimental Procedures

Twenty-eight Holstein heifer calves with a plasma protein (PP) content greater than 5.5 g/dl at 24 h of age were allotted randomly to one of four treatments: 1) 0 mg, 2) 1400 mg, 3) 2800 mg of dl-α-tocopheryl acetate 3 dissolved in a small quantity of water and given orally with a nipple bottle at weekly intervals, and 4) 1400 mg of dl-α-tocopherol 4 (vitamin E alcohol) given weekly by injection of equal doses in the gluteal muscles. Management was the same for all the calves. These procedures, including the ingredient and chemical composition of calf starter and the statistical analysis of data, are given elsewhere (26). Calves were on experiment until they were 12 wk old.

Experiment 1: Analytical Procedures

Plasma Protein and Packed Cell Volume: Jugular blood obtained at about 24 h after birth into heparinized blood collection tubes was used to determine plasma protein (PP) by refractometry 4 and packed cell volume (PCV) by a microhematocrit centrifuge. 5

Lymphocyte Blastogenesis Test. Jugular blood obtained at 2, 4, 6, 8, 10, and 12 wk of age into heparinized blood collection tubes was used within 1 h after collection. Two milliliters of blood mixed with 2 ml of phosphate-buffered saline (PBS) were layered on 4 ml of Histopaque-10776 containing ficoll and sodium diatrizoate, then centrifuged at 400 x g for 30 min. Lymphocytes sedimented at the interface between Histopaque-1077 and blood-PBS mixture were collected with a Pasteur pipette, washed twice with RPMI-1640 7 medium, and resuspended in RPMI-1640 containing 25 mM Hepes buffer and 10% fetal calf serum (FCS). Cells were counted in a hemocytometer, and the suspension was adjusted to contain 1 x 10⁶ cells/ml. The cell suspension lacking or containing 5 µg/ml phytohemagglutinin (PHA), was distributed in 200 µl/well in quadruplicate in a flat bottom tissue culture plate. This concentration of PHA was predetermined in several experiments in our laboratory to give maximum lymphocyte stimulation. Cultures were incubated at 37°C in a humidified carbon dioxide (CO₂) incubator. Viability of lymphocytes was > 95% during the course of the experiment as determined by trypan blue dye exclusion test. After 48 h of incubation, [³H] thymidine 8 (specific activity 6.7 Ci/mM) .2 µCi/culture was added. Cultures were harvested 24 h later on glass fiber filters in an automated cell harvester. 9 Filters were counted in a liquid scintillation counter 10 to determine the incorporation of [³H] thymidine in lymphocytes. Lymphocyte stimulation in-

---

3 Hoffmann-La Roche, Nutley, NJ 07110.
4 American Optical, Scientific Instruments Division, Buffalo, NY 14215.
5 International Equipment Co., Needham Heights, MA 02194.
6 Sigma Chemical Co., St. Louis, MO 63178.
7 Grand Island Biological Co., Grand Island, NY 14072.
8 Schwarz/Mann, Dickinson & Company, Orangeburg, NY 10962.
10 LS 6800 model, Beckman Instruments Inc., Fullerton, CA 92634.
Lymphoproliferative indices (LSI) were calculated as: LSI = Desolution per minute (DPM) of stimulated lymphocyte cultures/dpm of control cultures.

*Infectious Bovine Rhinotracheitis Virus Replication.* The effect of serum collected from experimental calves at 0, 2, 4, 6, 8, 10, and 12 wk of age on the in vitro replication of IBR virus was determined. Monolayer cultures of Madin-Darby bovine kidney (MDBK) cells were prepared in 35 x 10-mm tissue culture plastic dishes and maintained in Eagle’s minimal essential medium \(^{11}\) (MEM) with 10% FCS (IBR antibody-free). Cultures were washed twice with PBS and were inoculated with IBR virus at a multiplicity of .1 pfu/cell. After 1 h incubation at 37°C in a CO\(_2\) incubator, each set of cultures was maintained in MEM containing 10% serum from each experimental calf. The infected cultures were harvested 48 h postinoculation and frozen at -70°C. Infectivity titers were determined by tissue culture infective dose (TCID\(_{50}\)) method.

Frozen cultures were thawed, briefly sonicated, and serial log dilutions were prepared in MEM with 5% FCS (IBR antibody-free). Monolayers of MDBK cells grown in microtiter plates were infected with .2 ml of each serial log dilution (4 wells per dilution). Cells were observed for cytopathic effects 48 h postinfection, and TCID\(_{50}\) was determined by the method of Karber (15).

*Serum Immunoglobulins.* Serum IgG\(_1\), IgG\(_2\), and IgM were determined at wk 0, 6, and 12 by the single radial immunodiffusion (SRID) procedure with ready-to-use SRID assay kits. \(^{12}\) Experimental serum samples were diluted to 1:100 for IgG\(_1\), 1:15 for IgG\(_2\), and 1:3 for IgM, using .9% sodium chloride in distilled water before charging wells. Ring diameters were read after 24 h, and sample concentrations were determined by establishing a regression line for the ring diameter and concentration of known standards.

---

\(^{11}\) Gibco Laboratories, Grand Island Biological Co., Grand Island, NY.

\(^{12}\) V.M.R.D., Inc., P.O. Box 502, Pullman, WA 99163.

**Experiment 2**

The objective of this experiment was to study the effect of supplemental vitamin E on cell-mediated immune response of yearling heifers. Twelve Holstein heifers approximately 1 yr of age were given a single intramuscular injection of 2000 mg of dl-\(\alpha\)-tocopherol. \(^{3}\) Blood samples were obtained just before injection and 7 d later to determine serum \(\alpha\)-tocopherol and LSI. Serum \(\alpha\)-tocopherol was determined by high precision liquid chromatography (26) and LSI were calculated from the results of the lymphocyte blastogenesis test. Statistical analysis was by paired t test (27).

**Experiment 3**

This experiment studied the effect of in vitro addition of vitamin E on lymphocyte blastogenesis. Lymphocytes were isolated from blood of 10 unsupplemented calves used in Experiment 1 and the cultures were prepared for the lymphocyte blastogenesis test as described. Dl-\(\alpha\)-tocopherol \(^{6}\) dissolved in 95% ethanol and further diluted in RPMI-1640 medium was added to lymphocyte cultures in concentrations of 0, 1.25, 2.5, 5, 12.5, 25, and 50 \(\mu\)g/well (4 wells per concentration). The LSI was determined for each concentration of vitamin E. Statistical analysis of data was by one-way analysis of variance and then means separation by calculating the least significant differences.

**RESULTS**

**Experiment 1**

None of the calves was sick during the course of the experiment except for scours during the first few weeks after birth. Growth and metabolic responses are given elsewhere (26). Intramuscular injection of vitamin E produced at the site of injection a slight inflammatory swelling, which decreased with age. Results of PP and packed cell volume (PCV) determinations and LSI and serum immunoglobulins averaged across weeks are in Table 1. PP and PCV values were similar in all groups of calves.

LSI averaged across weeks were higher in calves given the high oral supplementation than in unsupplemented calves. Injected calves had higher values than all other calves. LSI
TABLE 1. Least square means of immune responses of calves given vitamin E.  

<table>
<thead>
<tr>
<th>Variable</th>
<th>Oral</th>
<th>Injection</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg</td>
<td>1400 mg</td>
<td>2800 mg</td>
</tr>
<tr>
<td>Plasma protein %</td>
<td>6.5</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Packed cell volume %</td>
<td>33.9</td>
<td>34.9</td>
<td>35.0</td>
</tr>
<tr>
<td>Lymphocyte stimulation index</td>
<td>44.9</td>
<td>64.1</td>
<td>77.2</td>
</tr>
<tr>
<td>Serum immunoglobulins (Ig)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1, mg/dl</td>
<td>1240</td>
<td>1456</td>
<td>1461</td>
</tr>
<tr>
<td>IgG2, mg/dl</td>
<td>145</td>
<td>135</td>
<td>165</td>
</tr>
<tr>
<td>IgM, mg/dl</td>
<td>44.8</td>
<td>52.3</td>
<td>70.7</td>
</tr>
</tbody>
</table>

Values at different weeks are presented in Figure 1. There were no significant differences between unsupplemented and orally supplemented calves at any of the weeks. Injected calves had significantly higher LSI than unsupplemented calves at 4 wk of age and higher values than all other calves at 8 wk of age.

Replication of IBR virus in cultures treated with serum from experimental calves showed a significant treatment × week interaction (P=.0002), and hence, the overall means and their differences were not calculated. Virus titers at individual weeks (Figure 2) were significantly lower with serum from calves given the high oral supplementation and injected calves at wk 12. A trend toward lower virus titers was observed using serum from supplemented calves as compared with serum from unsupplemented calves starting from wk 6, except for calves given lower oral supplementation at 8 wk of age. In addition, there was a trend toward progressive increase in virus titers with increasing age of calves.

Serum IgG1 and IgG2 averaged across weeks (Table 1) were similar in all groups. The IgM (Table 1) were higher in calves given high oral supplementation than in unsupplemented calves. The IgM concentrations at individual weeks are presented in Figure 3. Values were significantly higher at wk 6 in calves given the high oral supplementation than in unsupplemented calves.

EXPERIMENT 2

Serum α-tocopherol and LSI values of yearling heifers before and one wk after a single injection of 2000 mg of dl-α-tocopherol are presented in Table 2. Both serum α-tocopherol and LSI were significantly higher 1 wk after injection.

EXPERIMENT 3

The LSI values in response to in vitro addition of vitamin E to lymphocyte cultures are presented in Table 3. The values for cultures with different concentrations of vitamin E were not significantly different from control cultures without vitamin E.

Figure 1. Effect of supplemental vitamin E on the mean lymphocyte stimulation indices of calves. a,b,cMean within a week with different superscripts differ (P<.05).
Figure 2. Effect of supplemental vitamin E on the serum factors of calves that influence the replication of infectious bovine rhinotracheitis virus in tissue cultures. Virus titers were determined by tissue culture infective dose (TCID_{50}) method. Log TCID_{50} values were calculated by the method of Karber (15).

Means within a week with different superscripts differ (P<.05).

DISCUSSION

Mean PP concentrations at 24 h of age were similar in all groups, suggesting that all calves received similar and adequate amounts of colostrum. The immunological factors obtained through colostrum are Ig, complement, and lymphocytes (30). Very little is known about the mechanism of transport and functional significance of complement and lymphocytes present in colostrum. There is no placental transfer of Ig, and the newborn calf is dependent upon colostrum for passive immunity (4). The Ig of newborn calves appeared to be positively correlated with PP at about 24 h of age (11). Warner et al. (34) suggested that PP should be above 5.5% for satisfactory performance of calves.

Supplemental vitamin E for calves on typical diets enhanced their cell-mediated immunity, as suggested by higher LSI for calves given the high oral supplementation and in injected animals in both Experiment 1 and 2. Phytohemagglutinin is a specific mitogen for thymus-derived (T)-lymphocytes (17) and, hence, LSI mainly represents the cell-mediated immune response. Corwin and Gordon (7) reported that lymphocytes from mice supplemented with 5 mg vitamin E/100 g diet showed a 2.5 times increased response to mitogens, but supplementation with 50 mg/100 g diet increased the response by more than eightfold. Watson and Petro (35) reported a 2.7 times greater lymphocyte response to PHA in mice fed a high vitamin E diet for 4 wk than control mice but no significant differences in cell-mediated cytotoxicity. Lim et al. (23) reported enhanced antibody-dependent, cell-mediated cytotoxicity in mice fed a high vitamin E diet. Larsen and Tollenrud (22) also observed increased response to PHA with lymphocytes obtained from pigs supplemented with various amounts of α-tocopherol and sodium selenite for a period of 12 wk. A whole blood transformation assay used in their study indicated that vitamin E supplementation enhanced the mitogen response at all test additions of selenium.

Cipriano et al. (6) reported mean LSI of 76, 220, and 152 for calves on vitamin E-deficient diet, diet supplemented with 1 g of dl-α-tocopheryl acetate/d or normal unsupplemented
TABLE 2. Effect of injection of 2000 mg of dl-α-tocopherol on serum α-tocopherol and lymphocyte stimulation indices of yearling heifers.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before injection</th>
<th>After injection</th>
<th>SEM</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum α-tocopherol, µg/dl</td>
<td>179.2</td>
<td>354.2</td>
<td>27.1</td>
<td>.0001</td>
</tr>
<tr>
<td>Lymphocyte stimulation index</td>
<td>73.9</td>
<td>118.5</td>
<td>13.9</td>
<td>.0108</td>
</tr>
</tbody>
</table>

diet, respectively. However, the differences were not statistically significant, probably due to wide variation among and within calves at different times or to a very high amount of vitamin E used, which could have partially masked the enhancing effect. Detection of a significant response in the present study could have been due to a refined experimental procedure and to the repeated measures on the calves.

There are several possible reasons for higher blastogenic response to PHA in supplemented animals. The antioxidant action and prevention of peroxidative damage to the membranes of the cells and subcellular organelles such as mitochondria, microsomes, and lysosomes may be the most important mechanism. Lymphocytes normally have a higher concentration of free fatty acids (FFA) than other cells. Guinea pig spleen cells, for example, contain 14 to 17% FFA, whereas cells from viscera contain only 2 to 3% FFA (16). Lipid peroxidation and free radical attack may alter the membrane fluidity and result in changes in cell to cell and cell to substrate interaction and subsequent metabolic processes in lymphoid tissue and may be responsible for observed immunosuppression. Heinzerling (12) observed that in vitamin E-supplemented mice there is an increased accumulation of [3H]labeled dl-α-tocopheryl acetate in the thymus and spleen. Further, it was reported that rapid removal of labeled tocopherol nicotinate from the blood stream was accompanied by rapid deposition in the spleen, lungs, and liver, suggesting that the vitamin undergoes phagocytosis by the cells of the reticuloendothelial system (10, 31). This may be the reason for the higher blastogenic response of lymphocytes from injected calves in this experiment.

Another mechanism by which vitamin E may exert its effect on the immune system would be by controlling the synthesis of prostaglandins (18, 21, 29). The E₁ series of prostaglandins derived from dihomogamalinolenic acid play a major role in the regulation of thymus development and T-lymphocyte function (13). Some of the lymphocyte functions inhibited by prostaglandins include mitogen responsiveness (19, 24), cell-mediated cytotoxicity (13, 19), and lymphokine production (19). In vitro blastogenic response of calf lymphocytes to PHA was depressed when prostaglandin E₁ was added in concentrations ranging from $10^{-4}$ to $10^{-7}$ M (Reddy, unpublished data). Recently, Watson and Petro (35) observed lower serum corticosterone in mice fed a high vitamin E diet and concluded that lower serum corticosterone may explain some of the observations of enhanced T-lymphocyte activity, such as PHA-induced mitogenesis and antibody dependent cell-mediated cytotoxicity. Future research with calves should monitor the prostaglandin and

TABLE 3. Effect of in vitro addition of vitamin E on lymphocyte stimulation indices.

<table>
<thead>
<tr>
<th>α-Tocopherol, µg/culture</th>
<th>0</th>
<th>1.25</th>
<th>2.5</th>
<th>5.0</th>
<th>12.5</th>
<th>25.0</th>
<th>50.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte stimulation index¹</td>
<td>42.6</td>
<td>41.7</td>
<td>41.3</td>
<td>38.6</td>
<td>39.2</td>
<td>38.3</td>
<td>41.0</td>
</tr>
</tbody>
</table>

¹ There were no significant differences among means (P>.05).

Journal of Dairy Science Vol. 69, No. 1, 1986
serum corticosterone levels to determine possible reasons for higher PHA-induced lymphocyte blastogenesis.

The LSI of calves injected with dl-α-tocopherol were higher than those of calves given dl-α-tocopherol acetate orally. Although differences could be due to amount of serum α-tocopherol (26), they also could be due to the form of vitamin E given.

In vitro addition of vitamin E to lymphocyte cultures did not increase the blastogenic response to PHA. It is not clear whether this was due to the inhibitory effect of ethanol used to solubilize vitamin E, as opposed to the stimulatory effect of vitamin E, failure to homogenize effectively vitamin E in the medium, lack of enzymatic systems in vitro that may be necessary for the incorporation of vitamin E into lymphocytes, or higher than necessary dose of vitamin E.

Serum samples obtained after 6 wk of age from calves given supplemental vitamin E showed a trend toward inhibiting IBR virus replication. At wk 12, with serum from calves given the high oral supplementation and injected calves, the inhibition was significant as compared to that of unsupplemented calves. Viral titers were similar for all groups of calves until wk 6, probably because passive transfer of antibodies through colostrum was similar in all calves or because vitamin E did not alter other serum factors until wk 6. Vitamin E affects the levels of several enzymes (5) and hormones in the body (18). However, research is needed to study the effect of supplemental vitamin E on nonspecific factors of resistance such as interferon, C-reactive protein, β-lysin, lysozymes, and serum transferrins. Our results suggest that supplemental vitamin E may be useful for calves on conventional diets, at a stage when they are most vulnerable to respiratory diseases. Although there is no clear information on age-specific infection rate due to IBR virus, Langer (20) observed that the protective effect of maternally acquired antibody in calves affected the age distribution of infection rate. The persistence of colostrally acquired immunity varies from calf to calf; some calves lose it as early as 1 mo of age, but a few may have detectable maternal antibody at 6 mo of age (14). Results of the present experiment suggest that during this transition period between the loss of maternal antibody and the production of antibodies by the calf, supplemental vitamin E alters serum factors in such a way that there may be less morbidity due to a pathogen. Thus, in general, our results support the earlier findings (25, 33) that supplemental vitamin E may not only enhance humoral immune response but also possibly reduce morbidity and mortality due to pathogens.

Although there were no significant differences in IgG1 and IgG2 among treatments, there was a trend toward higher IgG1 concentrations in supplemented calves. It is not known whether this trend was due to less metabolic degradation of passively acquired antibody or enhanced synthesis by supplemented calves.

Calves given the high oral supplementation showed higher serum IgM concentrations than unsupplemented calves, suggesting their capability for a higher primary immune response, as IgM is the first class of antibodies to appear in the immune response to most antigens (17). The trend toward lower IgM concentrations in injected calves at wk 6 was probably due to an early IgM to IgG shift caused by inflammatory swellings due to injections of dl-α-tocopherol in the first few weeks. This is further supported by the fact that these calves also showed a trend toward higher IgG2 concentrations than other calves.

Conclusions

This study suggested that supplemental vitamin E enhanced both cell-mediated and humoral immune responses. It also altered serum factors in other ways, which may increase the possibility for protection against pathogens. Possibly these beneficial effects will be greater under more intensive management conditions. Further research with graded amounts of different forms of vitamin E given on a daily basis should determine the requirements of calves. Our data on immune responses of calves suggest that criteria for minimum requirements should not be based entirely on growth rate studies or on the amounts required to prevent clinical malnutrition deficiencies but also on the amounts needed to attain optimal health and immune competence.

REFERENCES

10 Frigg, M., and H. E. Gallo-Torres. 1977. Phago-
13 Horrobin, D. F., M. S. Manku, M. Oka, R. O. 
12 Heinzerling, R. H. 1974. The effect of vitamin E 
15 Karber, G. 1931. Beitrag zur kollektiven behandlung 
16 Kigoshi, S., and R. Ito. 1973. High levels of free 