CELLULAR IMMUNE RESPONSES IN PIGS FED A VITAMIN E- AND SELENIUM-DEFICIENT DIET

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ABSTRACT

The effects of dietary restriction of vitamin E (Vit E) and selenium (Se) on lymphocyte proliferation, natural killer (NK) cell activity, antibody-dependent cell-mediated cytotoxicity (ADCC), and on burst respiratory response of stimulated granulocytes as measured by chemiluminescence (CL) were studied in pigs. Six male weanling pigs were maintained for 25 d on a torula yeast-based diet containing no measurable amount of alpha-tocopherol and less than .02 mg of Se per kilogram of feed. Six others received the same basal diet supplemented with 33 IU of DL-alpha-tocopheryl acetate and .2 mg of Se per kilogram of feed. All pigs were inoculated with Salmonella typhiuis on d 21 of the feeding period and killed on d 25. Tests to measure cellular immune functions were performed on cells isolated from blood samples taken on d 21 and 25. After 21 d of feeding, lymphocyte blastogenesis responses to phytohemagglutinin, concanavalin A, and pokeweed mitogen in pigs fed the Vit E- and Se-deficient diet were normal compared with the response in pigs fed the supplemented diet. Moreover, the cytotoxic activity of NK cells, the ADCC response, and the CL response of granulocytes were not affected. After 25 d, a marked suppression of lymphocyte response to mitogens occurred in pigs fed the Vit E- and Se-deficient diet when the cells were cultured in the presence of autologous serum. When fetal bovine serum replaced autologous serum in the cultures, no suppression was observed. No effect on NK activity and ADCC was observed, whereas the CL peak response of granulocytes tended to be higher in pigs fed the deficient diet. Results suggest that the suppression of mitogen-induced lymphocyte proliferation is related to factor(s) present in serum from pigs fed a Vit E- and Se-deficient diet.

Key Words: Vitamin E, Selenium, Glutathione, Peroxidase, Lipid Peroxidation, Immune Response


Introduction

Both vitamin E (Vit E) and selenium (Se) are essential nutrients for humans and animals. They are involved in the protection of biological membranes against lipid peroxidation. Vitamin E, the major lipid-soluble antioxidant that is present in biomembranes, scavenges free radicals in the early stages of lipid peroxidation, and Se is essential to the activity of glutathione peroxidase (GSH-Px), which reduces H₂O₂ and lipid hydroxides to less reactive products (Hoekstra, 1975).

Disease problems associated with a Vit E and Se deficiency in weanling pigs have been reported (Van Vleet, 1980, 1982). Other
studies have also demonstrated that dietary supplementation of Vit E and/or Se increases the resistance against infectious diseases in mice (Heinzerling et al., 1974b), pigs (Teige et al., 1978, and chickens (Heinzerling et al., 1974a; Nockels, 1979). Although the relative importance of Vit E and Se in biological systems is not well defined, various studies have demonstrated that these nutrients exert a significant effect on the immune response (Sheffy and Schultz, 1979; Eskew et al., 1985; Jensen et al., 1988a). Thus, weanling pigs barrows weighing approximately 8 kg were randomly distributed into two groups. One group was fed a semisynthetic torula yeast diet containing less than .02 mg of Se/kg of feed and no measurable amount of alpha-tocopherol, and the other group received a deficient diet on lymphocyte response to heparinized tubes from the cranial vena cava of each pig on d 21 and 25.

Animals and Treatments. Twelve weanling barrows weighing approximately 8 kg were purchased from a specific-pathogen-free herd and placed in pens with a concrete floor. The pigs were randomly distributed into two groups. One group was fed a semisynthetic torula yeast diet containing less than .02 mg of Se/kg of feed and no measurable amount of alpha-tocopherol, and the other group received the same basal diet supplemented with .2 mg of Se/kg as sodium selenite and 33 IU of DL-alpha-tocopheryl acetate per kilogram of feed (Table 1).

Twenty-one days after the beginning of the experiment, all pigs were orally inoculated with Salmonella typhimurium at a concentration of $1 \times 10^9$ organisms in 10 ml of brain heart infusion (BHI) broth. Food was withheld of 12 h before inoculation. Four days after the inoculation, the pigs were killed. Twenty milliliters of blood were collected into heparinized tubes from the cranial vena cava of each pig on d 21 and 25.

Cell Preparation. Peripheral blood mononuclear cells were separated on a Ficoll-Hypaque gradient centrifugation as described by Boyum (1968). Briefly, blood was layered over the Ficoll-Hypaque with density of 1.077 and centrifuged at 600 x g for 30 min. After centrifugation, mononuclear cells were collected from the red blood cells by sedimentation of the red blood cells with 3% dextran. The contaminated erythrocytes were lysed with .83% buffered ammonium chloride solution. Then mononuclear cells and granulocytes were washed respectively three times in Hank’s balanced salt solution (HBSS) and resuspended in RPMI-1640 containing 10% of fetal bovine serum (FBS) or autologous serum (heat-inactivated at 56°C for 30 min) and 1% antibiotic solution (penicillin 100 U/ml, streptomycin 100 µg/ml, and fungizone .25 µg/ml). Cell viability was determined by the trypan blue exclusion technique (Mishell and Shiigi, 1980) and was always greater than 95%.

Lymphocyte Proliferation. Lymphocyte proliferation in response to mitogens was measured as described by Schultz and Adams (1978). Briefly, 50 µl of mononuclear cell suspensions ($5 \times 10^6$ cells/ml) were placed in wells of a 96-well microtiter flat bottom plate
previously filled with 100 µl of solution containing either phytohemagglutinin (PHA: 60 µg/ml)10, concanavalin A (Con A: 10 µg/ml)11, or pokeweed mitogen (PWM: 10 µg/ml)12. Plates were incubated at 39°C in a humidified, 5% CO2 air chamber for 72 h. Then 1 µCi of [3H]thymidine10 was added to each well, and plates were incubated for an additional 18 h. Cells were harvested onto glass fiber papers and incorporation of [3H]thymidine was determined in a scintillation spectrophotometer.11

Cytotoxicity Assay. A modified 51Cr-release microcytotoxicity assay protocol was performed as described by Yang and Schultz (1986) to measure ADCC and NK activities. Briefly, 1 to 2 × 10⁷ chicken red blood cells (CRBC) in .5 ml of RPMI-1640 containing 10% FBS were labeled with 150 µCi of 51Cr12 and used as targets in the ADCC assay. Labeled 51Cr-CRBC were adjusted to 1 × 10⁵ cells/ml in RPMI-1640 containing 10% inactivated FBS. To each of well of round-bottom microtiter plates, 100 µl of labeled target cells with or without porcine anti-CRBC were added. The porcine anti-CRBC has been previously produced (Yang, 1983) and was used at a dilution of 1/2,000. Then, granulocytes at effector to target ratios of 100:1, 50:1, or 25:1 in 100 µl were added. Each sample was tested in triplicate. The cells were incubated for 4 h at 39°C in a 5% CO2 incubator.

Human myeloblastic cell line K562 was used for target cells in the measurement of NK cell activity. Similar to CRBC, 1 to 2 × 10⁷ cells were suspended in .5 ml of culture medium and were labeled with 150 µCi 51Cr. Labeled 51Cr-K562 cells were adjusted to 1 × 10⁵ cells/ml in RPMI-1640 containing 10% of inactivated FBS and 100 µl were added into wells of round-bottom microtiter plates. Then effector cells were added at effector to target ratios of 100:1, 50:1, or 25:1. The cells were incubated for 18 h at 39°C in a 5% CO2 air chamber. All assays were performed in triplicate.

Control wells were also included in both assays. Target cells from each assay were either incubated with the medium alone for spontaneous release or with 3% Triton X-100 for maximum release. At the end of incubation, the plates were centrifuged at 200 g for 5 to 10 min at 100 µl of the supernatants were removed from each well for the determination of radioactivity in a gamma-counter.13 The spontaneous release never exceeded 10% of the maximal release. The following formula was used to calculate the percentage of specific lysis: % specific lysis = [(cpm in experiment – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release)] × 100.

Chemiluminescence Response. Granulocyte chemiluminescence response was assayed as described by Allen (1986) with a Packard Picolite Luminometer14. Cell suspensions were adjusted to 1 × 10⁵ cells/ube in 100 µl of HBSS containing 10% FBS, incubated in the luminometer at 39°C, and stimulated with the injection of 50 µl of ZAP TM14, a zymosan preparation containing luminol. Each sample was run in triplicate and the chemiluminescence response of each sample was measured over the next 40 min at 5-min intervals.

Vitamin E and Selenium Measurements. Vitamin E in blood plasma was assayed by HPLC as described by Bieri et al. (1979) with some modifications. In brief, 200 µl of plasma was mixed with 200 µl of ethanol and 50 µl of a solution of DL-alpha-tocopheryl acetate (100µg/ml), which were used as an internal standard. The mixture was stirred for 45 s and centrifuged, after 500 µl of N-heptane was added to it. Heptane extracts were evaporated using a Speed Vac Concentrator15. Each lipid extract was dissolved in 50 µl of diethyl ether followed by the addition of 150 µl of methanol. The sample was then injected into a Beckman HPLC instrument equipped with a 3.9-mm × 30-mm stainless steel column packed with micro Bondapak C-1816 and eluted with a 95% methanol:5% water solution. Peaks were detected with a Beckman variable wavelength UV detector set at 295 nm.

Serum Se concentration was measured by fluorometry, as described by Olson (1969). The activity of GSH-Px was determined in red blood cells as described by Mills (1959).
Statistical Analysis. All data were analyzed by one-way analysis of variance.

Results and Discussion

Pigs fed the Vit E- and Se-deficient diet had a lower concentration of Vit E in blood plasma than pigs fed the diet supplemented with Vit E and Se ($P \leq .01$; Figure 1). The level of Se in plasma was also significantly lower in pigs fed the deficient diet than in pigs fed the supplemented diet ($P \leq .001$; $0.21 \pm 0.02$ vs $0.131 \pm 0.007$). Selenium deficiency also impaired the GSH-Px activity in red blood cells ($P \leq .01$); after 14 d of feeding, the enzyme activity was $0.1009 \pm 0.015$ U/mg hemoglobin in pigs fed the deficient diet compared with $0.2034 \pm 0.021$ U/mg hemoglobin in the controls. Van Vleet (1982) reported similar results from pigs maintained on a Vit E- and Se-deficient diet.

Cellular immune response, as measured by in vitro lymphocyte response to mitogen stimulations, was lower in pigs fed a Vit E- and Se-deficient diet for 25 d. After 21 d of feeding, blood lymphocytes from pigs fed the Vit E- and Se-deficient diet and cultured in the presence of FBS or autologous serum proliferated similarly to lymphocytes isolated from the blood of pigs fed the supplemented diet (Figure 2). On d 25, 4 d after challenged with Salmonella typhimurium, lymphocyte responses to mitogens PHA, Con A, and PWM were significantly impaired in pigs fed the deficient diet when autologous serum was added to cultures (Figure 3). Similar results were obtained by adding suboptimal concentrations of PHA (30 μg and 15 μg/ml, results not shown). However, when cultures were supplemented with FBS, lymphocyte responses to mitogens were similar between groups. The suppressive effect seems to be related to the presence in the culture media of serum from pigs fed the deficient diet because lymphocyte proliferation was not affected when the cells were incubated in the presence of FBS. A previous study also showed that serum from dogs fed a Vit E- and Se-deficient diet suppressed dog lymphocyte proliferation (Langweiler et al., 1981).

In the present study, it was not expected that S. typhimurium would induce the appearance of a suppressive factor. In fact, cells from pigs fed the complete diet and infected with S. typhimurium responded well to mitogenic stimulations. However, it might be possible that in pigs maintained on the Vit E- and Se-deficient diet the injection of S. typhimurium may induce the formation of suppressive factors. Another study would be necessary to verify whether serum from uninfected, deficient pigs would
have the same effect on lymphocyte proliferation.

Cytotoxic activity of NK cells and ADCC activity, under the conditions of the present study, were not affected after feeding the Vit E- and Se-deficient diet for 25 d (Table 2). In comparison, Meeker et al. (1985) found, after a feeding period of 56 d with a Vit E- and Se-deficient diet, that NK activity was impaired but that ADCC activity was not affected in rats. Therefore, it seems that a feeding period of 25 d was not long enough to affect these cell functions in the present trial. However, in the NK assay, because of the presence of monocytes in cell suspensions, the cytotoxicity could be due in part to monocytes. But, monocytes could have also affected NK activity by secreting immunoregulatory molecules such as interferon-alpha. Consequently, the way that feeding pigs with a Vit E- and Se-deficient diet affected NK cell activity was not determined.

We used chemiluminescence responses to study the production of reactive oxygen species (ROS) by activated granulocytes in vitro. After 25 d of feeding, activated granulocytes isolated from pigs fed the deficient diet tended to have a higher peak response ($P \leq 0.05$).
0.10) than activated granulocytes from pigs fed the supplemented diet (Table 2). On the basis of these results, pigs fed the Vit E- and Se-deficient diets seemed to release more ROS than pigs maintained on the supplemented diet.

A Vit E- and Se-deficiency may have a severe effect on resistance to disease because of its suppressive effect on lymphocyte proliferation. In fact, deficiency of Vit E and Se has been associated with increased susceptibility to infections and depressed immune functions in several species. For instance, Teige et al. (1978) found that Vit E- and Se-deficient pigs inoculated with colonic and cecal material of pigs with dysentery showed more severe and more frequent clinical signs and a greater extent of enteric lesions than did control pigs. The mechanism by which serum from pigs fed a Vit E- and Se-deficient diet suppresses lymphocyte proliferation is not clear. What may be involved are changes in serum components and properties due to a lack of antioxidant. Both Vit E and Se are essential to prevent damage caused by oxidative reactions. Animals fed a Vit E- and Se-deficient diet had increased lipid peroxidation, as measured by ethane and pentane exhalation (Hafeman and Hoekstra, 1977) and plasma malondialdehyde concentration (Yoshioka et al., 1987). Lack of vitamin E and Se also caused an increase in the production of ROS in tissues, as measured by liver chemiluminescence response (Fraga et al., 1987).

We showed that the chemiluminescence response of activated granulocytes isolated from pigs fed the deficient diet tended to have a higher peak response than that from pigs fed the supplemented diet. Because of a reduction of radical scavengers and reduced activity of GSH-Px, the capacity of pigs maintained on a deficient diet to control formation of ROS may be altered and, consequently, the defense mechanisms against oxidative reactions may be impaired. This increase in chemiluminescence response could also be related to a reduced clearance of S. typhimurium. It has been demonstrated already that blood clearance of bacteria and carbon particles is increased by Vit E (Tengerdy et al., 1978). Then, reduced clearance of S. typhimurium in pigs fed the Vit E-

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**TABLE 2. CYTOTOXIC ACTIVITIES OF ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC) AND NATURAL KILLER (NK) CELLS AND CHEMILUMINESCENCE (CL) RESPONSE OF GRANULOCYTES LEUKOCYTES IN PIGS FED EITHER A VITAMIN E- AND SELENIUM-DEFICIENT DIET OR A COMPLETE DIET**

<table>
<thead>
<tr>
<th>Cell activity</th>
<th>Pigs on deficient diet</th>
<th>Pigs on complete diet</th>
<th>Day 21</th>
<th>Day 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCC E:T = 50:1</td>
<td>79.3 ± 1.7</td>
<td>78.7 ± 6.3</td>
<td>84.9 ± 3.9</td>
<td>82.2 ± 4.7</td>
</tr>
<tr>
<td>E:T = 25:1</td>
<td>71.6 ± 4.7</td>
<td>60.5 ± 10.7</td>
<td>83.3 ± 5.5</td>
<td>84.1 ± 5.5</td>
</tr>
<tr>
<td>NK E:T = 100:1</td>
<td>35.4 ± 5.0</td>
<td>31.7 ± 6.7</td>
<td>18.9 ± 5.9</td>
<td>18.6 ± 4.8</td>
</tr>
<tr>
<td>E:T = 50:1</td>
<td>31.4 ± 6.1</td>
<td>27.2 ± 7.5</td>
<td>9.0 ± 2.9</td>
<td>13.4 ± 4.1</td>
</tr>
<tr>
<td>E:T = 25:1</td>
<td>19.9 ± 7.7</td>
<td>20.5 ± 8.0</td>
<td>5.5 ± 1.7</td>
<td>6.4 ± 1.8</td>
</tr>
<tr>
<td>CL Peak</td>
<td>232.3 ± 84.2</td>
<td>127.0 ± 54.8</td>
<td>248.0 ± 31.8</td>
<td>188.1 ± 12</td>
</tr>
</tbody>
</table>

*Values are means ± SEM.

1E:T = effector:target ratio.

1Difference between deficient group and control group is significant at $P \leq .10$. 

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and Se-deficient diet might result in a greater synthesis of gamma-interferon, which enhances neutrophil respiratory burst activity (Berton et al., 1986).

Oxidative reactions and an abnormally high level of ROS have been shown to affect the integrity of biological membranes and the function of immunocompetent cells. For instance, it has been found that ROS suppresses mitogen-stimulated T-cell proliferation, mixed lymphocyte reaction, and antibody-dependent cell cytotoxicity (Grever et al., 1980; Zoschke and Staite, 1987). Decreased ability of cells to degrade H$_2$O$_2$ also impaired chemotaxis (McAllister et al., 1980), microbicidal activity (Serfass and Ganther, 1976), and lymphocyte response to mitogens (Parnham et al., 1983). Moreover, oxidation of cell thiol groups inhibited proliferation of lymphocytes in response to mitogen (Noelle and Lawrence, 1981) and cytotoxic activity of T lymphocytes (Redelman and Hudig, 1980).

We observed that lymphocytes from pigs fed the deficient diet responded to mitogenic stimulations such as well as lymphocytes from pigs fed the supplemented diet when FBS was added to the cultures (Figures 2 and 3). Other unpublished data from our laboratory showed that the suppressive effect caused by serum from pigs fed a Vit E- and Se-deficient diet was almost completely eliminated by the presence of FBS in the milieu. Furthermore, a study with dogs showed that the addition of Vit E or 2-mercaptoethanol to the culture media eliminated the suppressive effect caused by Vit E- and Se-deficient serum (Langweiler et al., 1983). One interpretation of all these results is that in animals fed a Vit E- and Se-deficient diet a high degree of lipid peroxidation might occur, and this may promote the release in the blood of factors capable of suppressing mitogen-induced blastogenesis.

In conclusion, weaning pigs fed a Vit E- and Se-deficient diet had impaired cell-mediated immunity as measured by lymphocyte response to mitogenic stimulations. This impairment of lymphocyte proliferation is related to the presence in the cultures of serum from Vit E- and Se-deficient pigs.

**Implications**

Results of the study showed that lymphocyte response to mitogenic stimulation and production of oxygen-reactive species by acti-

**Literature Cited**


