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Effects of vitamin E and selenium deficiencies on rat immune function

MARY LOU ESKEW, R. W. SCHOLZ, C. C. REDDY, DEBORAH A. TODHUNTER* & ARIAN ZARKOWER *Center for Air Environment Studies and the Department of Veterinary Science, The Pennsylvania State University, Pennsylvania, U.S.A.*

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Summary. The effects of dietary restriction of vitamin E and selenium were studied in male Long-Evans hooded rats. Weanling animals were maintained for 5–6 weeks on torula yeast-based diets, with or without the addition of vitamin E (150 IU/kg) or selenium (0·5 mg/kg), to form the following dietary groups: +E, +Se; +E, −Se; −E, +Se; −E, −Se, and a fifth group pair-fed with the −E, −Se group. This latter group exhibited a decreased rate of growth similar to the −E, −Se group. Lymphocyte blastogenesis in response to mitogens was decreased in animals fed the diets deficient in either vitamin E or selenium, and also in the pair-fed group. Very marked suppression of mitogen responses was seen in the doubly deficient group, as well as a greater loss of viability during culture. Spleen cell-mediated antibody-dependent lysis of chicken erythrocytes was increased in the doubly deficient group, although this difference could be abolished by the addition of catalase, but not indomethacin, to the culture medium. Dietary deficiency of vitamin E and selenium had no discernible effects on alveolar macrophage function, as measured by cell-mediated antibody-dependent cytolysis, killing of *Staphylococcus aureus* or regulation of T-lymphocyte blastogenesis.

INTRODUCTION

Vitamin E and selenium have been implicated in the protection of biological membranes against lipid peroxidation, a process that may be induced either by endogenously produced metabolic by-products such as free radicals and other oxidants, or by exposure to such atmospheric oxidants as ozone or nitrogen dioxide. Vitamin E is thought to act as a biological antioxidant, important in control of lipid hydroperoxide formation, by sequestering free radicals which initiate peroxidation (Diplock, 1978), whereas selenium, as an essential component of the enzyme glutathione peroxidase (Se-GSH-Px), functions as an antioxidant by reducing lipid hydroperoxides, once formed, to less reactive alcohols (Hoekstra, 1975).

Although the relative importance of vitamin E and selenium in biological systems is, to a great extent, undefined, various studies have found that the dietary levels of these nutrients exert a significant effect on the immune response (Corwin & Shloss, 1980; Heinzerling *et al.*, 1974; Spallholz *et al.*, 1973b). In early studies, Spallholz *et al.* (1973a) showed that dietary supplementation with non-toxic levels of selenium led to enhanced IgG and IgM antibody titres in mice. Subsequently, it was demonstrated that supplementation of selenium by diets, as well as by injections, resulted in an increase in the number of specific antibody-forming cells (Spallholz, *et al.*, 1973a, b; Spallholz *et al.*, 1975). Similarly, Tengerdy *et al.* (1973) reported that dietary supplementation with vitamin E leads to enhanced humoral immune responses in mice, while Tengerdy & Nockels (1973) reported similar effects in chickens. Also, vitamin E has been shown to offer protection against *E. coli* (Heinzerling *et al.*, 1974) and *Chlamydia* infections (Stephens, McChes-

* Present address: Department of Dairy Science, OARDC, Wooster, OH 44691, U.S.A.

Correspondence: Mary Lou Eskew, 102 Henning Building, University Park, PA 16802, U.S.A.

ney & Nockels, 1979). These studies indicate that selenium and vitamin E may play an important role in enhancing immune function.

It has been suggested (Baumgartner, 1979) that vitamin E and selenium modulate the immune response by protecting lymphocytes from the effects of various inhibitory products produced by phagocytes. These products, produced especially by macrophages, can include lipid hydroperoxides, superoxide anion, singlet oxygen, hydrogen peroxide, hydroxyl radical and prostaglandins. In addition to the deleterious effect of these reactive oxygen species, the immune system can be severely affected by alterations in the integrity of cellular membranes. Cellular membranes are involved in the release of these soluble substances, as well as being of great importance in the binding of mitogens and antigens, and binding and subsequent lysis of foreign cells by various cytotoxic effector cells.

Our studies were undertaken to examine the effects of vitamin E and selenium deficiencies, separately and together, on several aspects of the immune response. Important to this discussion is the fact that very little information is available on the effects of depressed antioxidant capability on alveolar macrophage function. Although some work has been done on the effects of either vitamin E deficiency or selenium deficiency alone, very little is known of combined deficiency states. It is well known that biological systems often have compensatory mechanisms to enable their adaptation to adverse conditions. The imposition of the doubly deficient diet might result in an even greater demand on the body's ability to cope with oxidant stress and, thus, lead to a better understanding of the limits of this compensation.

MATERIALS AND METHODS

Animals and diet

Weanling male Long-Evans hooded rats (Charles River Co., N. Wilmington, MA) were divided into four groups and placed on chemically defined, torula yeast-based diets for a period of 5 weeks. The composition of the basal diet (−E, −Se) was formulated to provide adequate levels of all known nutrients needed by rats (other than vitamin E and selenium). The basal diet contained less than 0·01 mg/kg selenium and undetectable vitamin E levels. Three additional diets (+E, −Se; −E, +Se; and +E, +Se) were included to establish a 2×2 factorial arrangement of treatments. Selenium was supplemented as sodium selenite (0·5 mg/kg) and vitamin E as δ-α-tocopherol acetate (150 IU/kg). Fat supplied 36% of the total calories in these diets. Deficiencies in vitamin E and selenium were ascertained for each experiment by measurement of rat plasma and liver microsomal tocopherol, blood selenium and liver glutathione peroxidase.

Pair-feeding experiments

In our preliminary experiments, we found that rats which were fed on diets deficient in *either* selenium or vitamin E showed no change in weight gain or superficial appearance of good health, whereas rats fed on diets deficient in *both* vitamin E and selenium appeared sick, decreased their food intake and began to lose weight by the fourth week.

Since malnutrition and starvation are known to affect the immune response (McFarlane, 1976; Weindruch *et al.*, 1979), an additional group, pair-fed with the −E, −Se group using the diet supplemented with vitamin E and selenium, was added to several experiments to ascertain that changes seen were due to deficiencies in vitamin E and selenium, and not to a decrease in dietary intake.

Cell preparation

Rats were anesthetized with sodium pentobarbital. Spleens were removed aseptically and teased in Hanks' balanced salt solution (HBSS, Gibco, Grand Island, NY). Following centrifugation and enumeration of cells with an electronic counter (Coulter Electronics Inc., Hialeah, FL), cell suspensions were adjusted to appropriate concentrations in RPMI 1640 (Gibco) containing 10 mM HEPES buffer (Gibco). Lungs were first perfused with sterile physiological saline via the right ventricle to remove blood from the pulmonary vasculature. A tracheal cannula was inserted and the lungs were lavaged with phosphate-buffered saline (Ca^{2+} and Mg^{2+} free) in 5 ml amounts, until a total volume of 30 ml had been collected. Following centrifugation, cells were adjusted to appropriate concentrations using RPMI 1640. Cells were counted by haemocytometer. Differential smears were made using a Cytospin (Shandon Instruments, Sewickley, PA) and stained with Diff-Quik (Harleco, Gibbstown, NJ).

Response to mitogen stimulation

Cultures were prepared in 96-well U-bottomed microculture plates (Flow Laboratories, McLean, VA) and contained 2×10^5 spleen cells in a total volume of 0·15

ml RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Garamycin (Schering Corp., Kenilworth, NJ). Optimal concentrations of mitogens were used. Final concentrations in the wells were: concanavalin A (Con A, Miles Laboratories, Kankakee, IL) 13·3 μg/ml, phytohaemagglutinin–P (PHA, Difco Laboratories, Detroit, MI) 1·3 μl/ml, or *E. coli* (055:B5) lipopolysaccharide, boivin-extracted (LPS, Difco) 33·3 μg/ml. After 48 hr of incubation, the cultures were labelled with 0·5 μCi of [^{3}H]thymidine (S.A. 6·7 Ci/mmole, New England Nuclear, Boston, MA) and, 24 hr later, were harvested on glass fibre filters using an automatic cell harvester (Brandel Laboratories, Rockville, MD). Samples were prepared for counting by solubilization in Protosol and suspending in Econofluor scintillation fluid (New England Nuclear). Results were expressed as counts per minute ($\log_{10}$).

Antibody-dependent lysis of chicken erythrocytes (CRBC)

The method used was a modification of the procedures reported by Berger & Amos (1977) and Clark & Klebanoff (1977). The CRBC were freshly collected each day, washed in HBSS and labelled by incubation of 2×10^7 cells with 100 μCi of sodium ^{51}Cr-chromate (S. A. 200–500 Ci/g) for 2 hr at 37°. After extensive washing to remove the unincorporated isotope, the erythrocytes were coated with diluted rabbit anti-CRBC serum (1:160) or diluted normal rabbit serum. For spleen cultures, varying numbers of the effector cells were added, followed by addition of the antisera or normal serum-coated target cells (2×10^4/well). For the pulmonary washout cells, varying numbers of cells, primarily macrophages, were allowed to adhere to the microculture plate for 2 hr. Following washing to remove the non-adherent cells, the antisera or normal serum-coated CRBC were added. In both instances, the incubation time was 4 hr. At this time, the plates were centrifuged and the supernatant removed for gamma counting. Both spontaneous and total release were determined. The results were expressed as follows:

$$\text{percentage lysis} = \frac{(\text{c.p.m. test release} - \text{c.p.m. normal rabbit serum release})}{(\text{c.p.m. total release} - \text{c.p.m. normal rabbit serum release})} \times 100.$$

The spontaneous release values were essentially the same as those for the normal rabbit serum release.

Adherent cell regulation of T-cell mitogenesis

Varying numbers of alveolar washout cells were allowed to adhere to flat-bottomed microculture plates. After 2 hr the non-adherent cells were removed by washing, and 2×10^5 normal rat spleen cells were added followed by optimal doses of either Con A or PHA. After 24 hrs of incubation, cultures were labelled with [^{3}H]thymidine and harvested as described in the above procedure for response to mitogen stimulation.

Bactericidal assay

The method used was essentially that of Quie *et al.* (1967) as modified by Zeligs, Nerurkar & Bellanti (1977). Equal numbers (2×10^5) of *Staphylococcus aureus (S. aureus*, Newbould strain) and alveolar washout cells were mixed in sterile Bio Vials (Beckman, Irvine, CA) in 1 ml RPMI 1640 containing 10% normal rabbit serum (Gibco) for opsonization. An aliquot was removed at the beginning and again after 2 hr of incubation, diluted in distilled water to lyse the lung cells, and plated using Trypticase soy agar (BBL, Cockeysville, MD). Data were presented as the percentage of *S. aureus* remaining after 2 hr incubation.

Statistical analysis:

For statistical analysis, mitogen data were logarithmically transformed to better approximate a normal distribution. Analysis of variance and Duncan's multiple range test were used to test for both factor effects and differences between groups, using the SAS statistical analysis system (SAS Institute Inc., Cary, NC). Differences in treatments were judged to be statistically significant at $P < 0{\cdot}05$.

RESULTS

Effect of diets on rat body weight gain

It can be seen from Fig. 1 that a deficiency of either vitamin E or selenium caused no change in body weight gain. However, with the rats placed on diets deficient in both of these nutrients, food consumption and subsequent weight gain decreased. The rats pair-fed with the doubly deficient group showed a similar decline in weight gain.

Effect of vitamin E and selenium deficiency and pair-feeding on spleen lymphocyte response to mitogen stimulation

The effect of vitamin E and selenium deficiency and

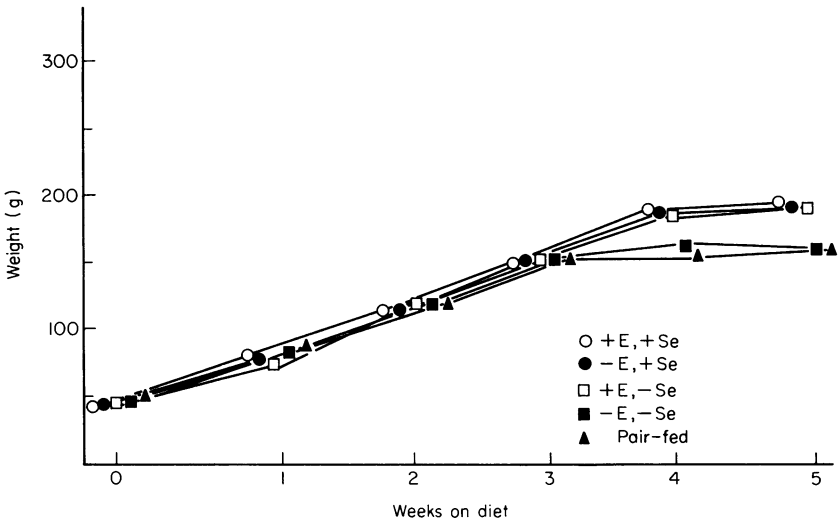

Figure 1. Effect of diet on body weight gain (n = eight or nine rats per group.

Table 1. The effect of vitamin E and selenium deficiency and pair-feeding on spleen lymphocyte response to mitogen stimulation

Mitogen	E	Se	N	Mean c.p.m. ($\log_{10}$) ± SE
Con A	+	+	8	4·66 ±0·117*
	−	+	8	4·166±0·105†
	+	−	8	4·151±0·127†
	−	−	8	3·520±0·033‡
	Pair-fed		9	4·296±0·056†
PHA	+	+	8	4·634±0·077*
	−	+	8	4·438±0·084*†
	+	−	8	4·136±0·214†
	−	−	8	3·423±0·134‡
	Pair-fed		9	4·485±0·071*†
LPS	+	+	8	3·886±0·071*
	−	+	8	3·699±0·088*
	+	−	8	3·737±0·122*
	−	−	8	3·381±0·075†
	Pair-fed		9	3·776±0·063*
None	+	+	8	2·941±0·061*
	−	+	8	2·731±0·079*
	+	−	8	2·748±0·146*
	−	−	8	2·358±0·059†
	Pair-fed		9	2·719±0·110*

Treatment means with different footnotes are significantly different ($P < 0·05$) within mitogen groups.

Table 2. Effect of vitamin E and selenium deficiency on the viability of rat spleen cells in culture

	% viable cells ($\bar{x} \pm$ SE)			
	Dietary treatment			
Time (hr)	+E, +Se	−E, +Se	+E, −Se	−E, −Se
0	95·6±0·68*	96·1±0·83*	96·2±0·84*	97·1±0·78*
24	80·8±2·63*	74·3±2·76*	75·2±3·30*	74·9±3·29*
48	68·2±5·20*	67·3±3·83*	65·8±3·32*	63·0±3·58*
72	52·5±7·14*	53·3±3·86*	62·8±4·20*	39·8±4·39†

Means within a horizontal row with different footnotes are significantly different ($P < 0.05$).
n = five rats per group.

pair-feeding on splenic response to mitogen stimulation can be seen in Table 1. When the data were analysed for factor effects (excluding the pair-fed group), it was seen that either vitamin E or selenium deficiency resulted in decreased thymidine incorporation in both mitogen-stimulated and non-stimulated cultures. In the cultures stimulated with PHA, vitamin E and selenium acted synergistically to produce an even greater decrease. When analysed for differences between groups, pair-feeding as well as deficiencies of either selenium or vitamin E resulted in small decreases in response, significant only with the T-cell mitogens PHA and Con A. Obviously, great depression of thymidine incorporation was seen in all responses of the doubly deficient group.

Effect of vitamin E and selenium deficiency on the viability of rat spleen cells in culture

As a decrease was recorded in mitogen responsiveness in the vitamin E and selenium-deprived animals, an experiment was carried out to measure possible alterations in viability during the 3-day culture period. Viability was determined on unstimulated rat spleen cells by trypan blue exclusion using cultures consisting of 2×10^5 cells in RPMI 1640 containing 10% fetal bovine serum. The data are shown in Table 2. The initial viabilities were the same in all the dietary groups. Over the 3-day culture period, there was a decline in viability in all groups and, by the third day of culture, the doubly deficient group showed a significantly greater loss in viability than the other three groups.

Effect of vitamin E and selenium deficiencies and pair-feeding on spleen antibody-dependent lysis of chicken red blood cells

As can be seen in Table 3, the feeding of diets deficient in both vitamin E and selenium resulted in an increase (significant at the highest effector:target ratio) in the ability of spleen cells to mediate lysis of CRBCs. Food restriction had no effect on lytic ability.

Table 3. Effect of vitamin E and selenium deficiency and pair-feeding on spleen antibody-dependent cytolytic activity

			Mean % lysis ± SE		
E	Se	N	100:1	50:1	10:1
+	+	8	32·3±3·6*	29·0±4·0*	14·0±3·5*
−	+	8	33·4±2·5*	30·8±2·6*	11·9±1·7*
+	−	7	32·7±3·2*	28·5±4·1*	10·3±1·9*
−	−	9	44·7±3·2†	37·7±3·8*	15·2±1·6*
pair-fed		8	28·6±3·1*	26·6±2·8*	8·2±1·1*

Treatment means with different footnotes are significantly different ($P < 0.05$) within the same E:T ratio.

Effect of catalase and indomethacin on antibody-dependent cytolytic ability

Since both prostaglandins and oxygen intermediates such as hydrogen peroxide have been implicated as being important in the regulation and mediation of cytolytic activity (Antonaci *et al.*, 1982; Nathan,

Table 4. Effect of catalase and indomethacin on antibody-dependent lytic ability

	Mean % lysis ± SE	
	Dietary treatment	
Addition to culture	+E, +Se	−E, −Se
None	29·2 ± 2·5	40·9 ± 2·7*
Indomethacin	29·4 ± 3·2	43·6 ± 2·6*
Catalase	25·9 ± 4·4	31·2 ± 2·0
Indomethacin + catalase	29·0 ± 3·6	33·4 ± 3·4

Catalase was added to make a final concentration of 400 U/ml. The final concentration of indomethacin was 10 μg/ml. The effector to target cell ratio was 100:1.

n = four rats per group.

* Significantly different from the +E, +Se group ($P < 0·05$).

1982), an experiment was performed to see if alteration of these metabolites by catalase, a scavenger of hydrogen peroxide, or indomethacin, a classical inhibitor of prostaglandin synthesis, would affect target cell lysis. As shown in Table 4, the addition of indomethacin did not seem to have an effect on target cell lysis in either dietary treatment group. The addition of catalase, on the other hand, seemed to preferentially decrease the cytolytic ability of the group deficient in both selenium and vitamin E.

Effect of vitamin E and selenium deficiencies on alveolar macrophage function

Deficiencies of vitamin E and selenium had no effect on the numbers or types of cells recovered by pulmonary lavage (Table 5). Likewise, it was found that alveolar macrophage function, as measured by anti-

Table 5. Effect of vitamin E and selenium on the number and type of cells recovered by pulmonary lavage

	Mean no. cells recovered* ($\times 10^5$) ± SE			
	+E, +Se	−E, +Se	+E, −Se	−E, −Se
Total	22·1 ± 5·9	16·3 ± 1·6	18·5 ± 2·9	17·2 ± 3·9
Macrophages	19·9 ± 5·3	14·7 ± 1·6	15·1 ± 2·6	15·6 ± 3·7
Lymphocytes	1·6 ± 0·6	1·3 ± 0·3	1·9 ± 0·7	0·8 ± 0·2
Neutrophils	0·4 ± 0·3	0·1 ± 0·0	0·2 ± 0·1	0·7 ± 0·2

* n = eight rats per group.

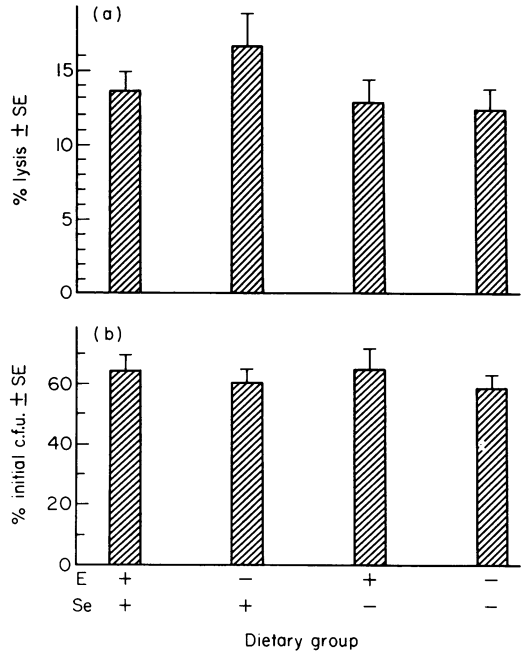

Figure 2. Effect of vitamin E and selenium on destructive activities of alveolar macrophages (n = eight rats per group). (a) Effect on ability to mediate antibody-dependent lysis of chicken erythrocytes; (b) effect on bactericidal activity.

body-dependent lytic ability (Figure 2a), bactericidal activity against *S. aureus* (Figure 2b) or the ability to regulate the proliferative response of lymphocytes to mitogens (Figure 3), was not affected by dietary deficiencies of vitamin E and/or selenium.

DISCUSSION

The results we have reported here confirm the depressive effects of vitamin E and selenium deficiency on immune function, as assessed by lymphocyte proliferative response to mitogens. This is especially evident in the markedly reduced responses of the doubly deficient rats to T-cell mitogens, particularly PHA. Although it appears that diminution of food intake, as assessed by the pair-feeding experiments, contributes to this decrease, the actual extent of this effect would seem to be small in relation to the magnitude of the depression in the group deprived of both vitamin E and selenium. The mitogen response in the doubly deficient animals must also be adversely affected by the greater loss of viability in culture of spleen cells from

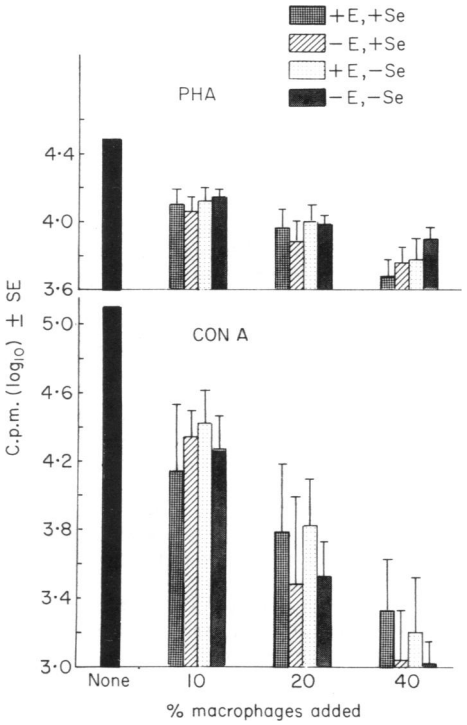

Figure 3. Effect of vitamin E and selenium on the ability of alveolar macrophages to regulate the proliferative response of lymphocytes to mitogens. (n = eight rats per group).

these animals. Additionally, Langweiler, Schultz & Sheffy (1981) found that serum from vitamin E-deficient dogs contains a factor capable of greatly suppressing mitogen-induced blastogenesis. Such a suppressive factor could also be affecting mitogen responses in our studies, since the lymphocytes were not extensively washed prior to culture.

In previous experiments in our laboratory, using mice, a deficiency of both vitamin E and selenium had no effect on spleen antibody-dependent cytolytic activity, although it did influence other cell-mediated lytic reactions, causing severely depressed natural killer activity and T-cell mediated cytotoxicity (H. C. Meeker, M. L. Eskew, W. Scheuchenzuber, R. Scholz and A. Zarkower, manuscript in preparation). Lim *et al.* (1981) reported that although ingestion of vitamin E at high levels resulted in an increase in antibody-dependent cytotoxic activity, prolonged use eventually led to a significant decrease. In our studies, we have found that severe antioxidant deficiency, i.e. lack of both selenium and vitamin E causes an increase in antibody-dependent cell-mediated cytotoxic activity. This increase is not too surprising in the light of the fact that excess production of reactive oxygen metabolites, such as hydrogen peroxide, hydroxyl radical or other oxygen intermediates, is believed to be involved in mediating cytotoxic reactions (Nathan, 1982). Our experiments indicate that excess hydrogen peroxide production may be the cause of the increase in antibody-dependent activity seen in the doubly deficient groups, since catalase abolished the difference between the two groups. It would seem, though, that hydrogen peroxide is not the major mediator of the normal antibody-dependent cytolytic event, since catalase had such a small effect in decreasing the cytolysis in the selenium and vitamin E supplemented group. As in other cell-mediated cytolytic reactions, direct contact or binding had to occur since no lysis above background was seen in the cultures containing normal serum, but only in those containing specific antisera directed against the chicken erythrocyte targets. Unlike the effect on mitogenic responses, pair-feeding had no effect on antibody-dependent cell-mediated cytotoxicity.

Most surprising of all, perhaps, is the lack of effect of selenium and vitamin E deficiency on alveolar macrophage function, as measured by antibody-dependent lysis of chicken erythrocytes, killing of *S. aureus* or regulation of lymphocyte mitogenic responses. All of these functions are thought to be regulated or mediated, at least to some extent, by macrophage production and secretion of intermediates of oxygen and arachidonic acid metabolism, such as hydrogen peroxide, singlet oxygen, prostaglandins etc. (Nathan, 1982; Metzger, Hoffeld & Oppenheim, 1980). The results of our experiments suggest that the macrophage functions measured in this study are relatively unaffected by alteration of antioxidant mechanisms, or that resident lung macrophages have compensatory mechanisms for maintaining their functional integrity under adverse conditions of oxidant stress. Although alterations in macrophage activity were not seen in these experiments, conditions resulting in macrophage activation, or exposure to environmental pollutants such as ozone or nitrogen dioxide, might create sufficient excess oxidant stress to lead to severe compromise of function in the vitamin E, selenium deficient animal.

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