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Vitamin E Decreases Superoxide Anion Production by Polymorphonuclear Leukocytes

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ABSTRACT. Pharmacologic serum levels of vitamin E administered to low birth weight infants predispose them to infectious complications. We studied *in vitro* the effect of vitamin E, its vehicle and buffer (Krebs Ringers phosphate glucose) on the ability of human polymorphonuclear leukocytes (PMN) to produce superoxide anion, an oxygen radical important for bacterial killing. We found that superoxide anion production after a 5-min exposure to phorbol myristate acetate was significantly decreased in vitamin E-treated PMN (76 ± 15 nM/10^7 PMN) compared to vehicle-treated PMN (289 ± 109 nM/10^7 PMN). We also found that significantly decreased superoxide anion production was associated with 5.0 and 10.0 mg/dl but not with 3.5 mg/dl vitamin E. Our results support the hypothesis that pharmacologic concentrations of vitamin E depress PMN oxidative activity. (*Pediatr Res* 23: 245–248, 1988)

Abbreviations

PMN, polymorphonuclear leukocytes
AT, *dl*-α-tocopherol
V, vehicle
KRPG, Krebs-Ringers-phosphate-glucose
LDH, lactate dehydrogenase
PMA, phorbol myristate acetate
NEM, N-ethylmaleimide

Vitamin E, or α-tocopherol, is administered to many low birth weight infants in an attempt to decrease the incidence of severe retinopathy of prematurity (1–3) intraventricular hemorrhage (4–6), and possibly mortality (5–7). Recently, pharmacologic levels of vitamin E (5 mg/dl) have been associated with an increased risk for sepsis and necrotizing enterocolitis (8). We and others have hypothesized that this increased risk for infectious complications may be related to altered host defenses, specifically polymorphonuclear leukocyte oxygen radical production (8).

In vitro, pharmacologic levels of vitamin E have been associated with decreased PMN chemotaxis, random migration, and bactericidal activity (9–11). Decreased oxygen radical production by PMN has also been found in the presence of high vitamin E levels. Baehner *et al.* (11). demonstrated decreased hydrogen peroxide production but normal superoxide anion production. Shigeoka *et al.* (12) found that PMN from stressed neonates and treated with high concentrations of vitamin E responded to stimuli with significantly less chemiluminescence (a measure of total oxygen radical generation). To further investigate the depressant effect of pharmacologic levels of vitamin E on PMN oxidative metabolism we have studied superoxide anion production *in vitro* by PMN exposed to pharmacologic levels of vitamin E.

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MATERIALS AND METHODS

Chemicals. Vitamin E (AT) and its V were graciously supplied by Hoffmann-LaRoche, Inc., Nutley, NJ. The composition of the preparation is identical to that given to neonates in whom an increased risk for sepsis and necrotizing enterocolitis was identified (Table 1). The free tocopherol was used in our experiments because it is the form of vitamin E found in the serum and tissues after administration of various formulations of vitamin E (*i.e* tocopherol acetate, tocopherol succinate). KRPG (pH 7.4) was the buffer utilized for cell preparation and during the superoxide anion assay. Potassium phosphate buffer (pH 7.5) was used during the LDH assay. Superoxide dismutase cytochrome C, PMA, NEM, pyruvic acid, nicotinamide adenine dinucleotide, calcium chloride, sodium chloride, magnesium chloride, disodium monophosphate, and potassium chloride were obtained from Sigma Chemical Company, St. Louis, MO. Sodium azide was obtained from Fisher Scientific, Cincinnati, OH.

Collection and separation of PMN. Venous blood was obtained from healthy adult donors according to the institution's informed consent policy. Heparin (10 U/ml) was used as the anticoagulant. PMN were isolated by Ficoll-Hypaque density centrifugation according to the method of Boyum (13) as modified by Ingraham et al. (14). PMN were resuspended in KRPG to a concentration of 1×10^7 PMN/ml. After isolation the PMN were more than 95% viable by Trypan blue exclusion; LDH release was consistently less than 8%.

Assays. Superoxide anion was measured according to the superoxide dismutase-inhibitable reduction of cytochrome C (15). Lactate dehydrogenase was measured by the method of Weening et al. (16). During the PMA exposure time study, a Pye-Unican spectrophotometer (SP-1800) was used for both assays. A Gilford response single beam spectrophotometer was used during the Vitamin E dose response study.

Protocol. Immediately after PMN collection and a 10-min incubation period at 37° C, PMN (250 μl of 1×10^7 PMN/ml) were added to 3 ml polypropylene test tubes containing cytochrome C (119 μM), sodium azide (1 mM), and KRPG (amount to achieve total volume of 0.5 ml). Superoxide dismutase (48 μg) was used as a control and all studies were done in triplicate. Vitamin E (3.5 mg/dl) or vehicle (equivalent concentration to that in which the vitamin E is suspended) was added and incubated in a shaking water bath for 10 min at 37° C. As part of the dose-response study, the cells were washed after treatment with vitamin E, vehicle, or KRPG and resuspended in buffer. The cell suspensions were then exposed to 50 ng/ml PMA. Initial

Table 1. *Vitamin E preparation*

AT	50.0 mg
Ethyl alcohol 100 proof	0.1 ml
Propylene glycol	0.1 ml
Benzyl alcohol	0.01 ml
Sodium acetate trihydrate	0.3 mg
Glacial acetate acid	9.0 mg
Disodium acetate	0.1 mg
NaOH solution (10%, to achieve pH 4 ± 0.1)	
Water for injection (to achieve final volume of 1.0 ml)	

PMA exposure time studies were for 1, 5, and 10 min. The PMA exposure time in subsequent vitamin E dose-response studies (3.5, 5.0, and 10.0 mg/dl vitamin E) was 5 min. After the specified exposure time to PMA, the reduction of cytochrome C was stopped by addition of ice-cold NEM (0.5 ml). The tubes were centrifuged (400 × *g*, 5 min, 4° C) and supernatant absorptions read spectrophotometrically at 550 nM. The results were converted to nM reduced cytochrome C/10^7 PMN using the extinction coefficient of 19.1×10^3 M^{-1} cm^{-1}. Supernatants were also assayed for LDH by adding 0.200 ml to a cuvette containing 0.850 ml pyruvic acid (0.34 mM in potassium phosphate buffer, pH 7.5) and 0.025 ml NADH (potassium phosphate buffer, pH 7.5). The OD at 340 mM was observed continuously for 5 min at 25° C. Slopes of the linear segment of the curve generated were compared to that generated for supernatants of PMN disrupted by sonication (three 10-s bursts with 10-s rest intervals, 0° C; Heat systems, Ultrasonics, Inc., Farmingdale, NY, model W225, output setting of 3).

Data analysis. Statistical comparison of data was performed using analysis of variance with either multiple comparison or the least significant difference for a specific pair of means. Significance was defined at the $p < 0.05$ level.

RESULTS

PMA exposure time study. Superoxide anion production after exposure to PMA for 0, 1, 5, and 10 min is depicted in Figure 1. The amount of superoxide anion produced by 5 min was significantly less in the vitamin E-treated PMN (76 ± 15 nM/10^7 PMN) compared to the vehicle-treated PMN (286 ± 109 nM/10^7 PMN). This trend continued through 10 min of PMA exposure with the amount of superoxide anion produced by the vitamin E-treated PMN being 226 ± 18 nM/10^7 PMN *versus* 502 ± 174 nM/10^7 PMN produced by vehicle-treated PMN ($p < 0.05$).

LDH release from vehicle-treated PMN was twice that from vitamin E-treated PMN at all PMA exposure times (Fig. 2). After incubation for 10 min with vehicle and immediately before PMA exposure (time 0, Fig. 2), vehicle-treated PMN released 25 ± 14% LDH whereas vitamin E-treated PMN released 10 ± 6% LDH. LDH release increased with PMA exposure so that by 5 min, 42 ± 10% LDH was released from vehicle-treated PMN. At the same PMA exposure time, vitamin E-treated PMN released only 23 ± 7% LDH. LDH release from untreated PMN exposed to PMA for 5 min was 11 ± 4.5%.

Vitamin E dose-response study. Because of the significant difference in superoxide anion production between vitamin E and vehicle-treated PMN, we performed a dose-response curve using vitamin E concentrations which were demonstrated *in vivo* to increase the risk of infectious complications in premature infants (8). In the presence of 3.5 mg/dl vitamin E, no significant difference in superoxide anion production by buffer-treated PMN was found (Fig. 3). At 10.0 mg/dl, the amount of superoxide anion produced was significantly less from the vitamin E-treated PMN (108 ± 62 nM/10^7 PMN) compared to buffer controls (325 ± 109 nM/10^7 PMN) and vehicle-treated PMN

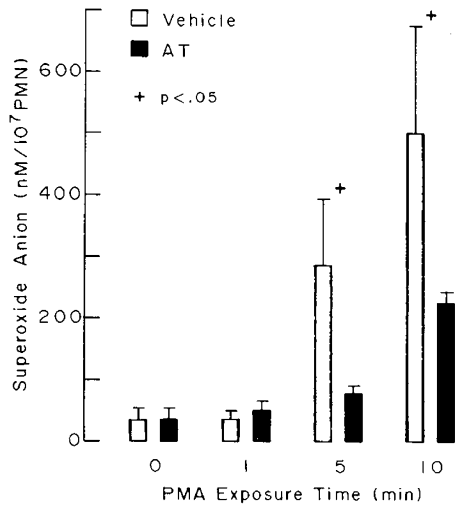

Fig. 1. Superoxide anion production—PMA exposure time study. The amount of superoxide anion produced (nM/10^7 PMN) in the presence of vitamin E (AT) or vehicle is compared for 0, 1, 5, and 10 min of PMA exposure. The *bars* depict the mean ± 1 SD from seven experiments. Superoxide anion production is significantly lower by the AT-treated PMN at 5 and 10 min ($p < 0.05$).

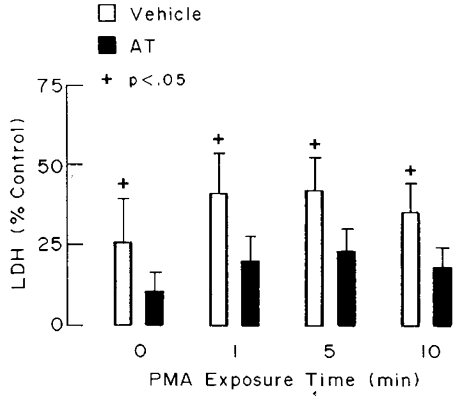

Fig. 2. LDH release—PMA exposure time study. The amount of LDH released at 0, 1, 5, and 10 min is significantly lower at all time points from PMN treated with vitamin E (AT) compared to vehicle ($p < 0.05$). The *bars* depict mean ± 1 SD from seven experiments.

(562 ± 89 nM/10^7 PMN). The amount of superoxide anion produced at 5.0 mg/dl vitamin E was intermediate between that at 3.5 and 10.0 mg/dl and not significantly different from buffer-controls; however, the amount produced (258 ± 63 nM/10^7 PMN) was significantly less than that of the vehicle-treated PMN (548 ± 85 nM/10^7 PMN).

LDH release at all three concentrations of vitamin E and vehicle were significantly elevated compared to buffer-treated PMN (Fig. 4). Because elevations in LDH release is associated with cell death that could account for diminished superoxide anion production, we incorporated a wash step into our protocol to remove vitamin E and vehicle from the solution surrounding the PMN before PMA exposure. LDH release was decreased to levels less than 12% in the buffer and vitamin E-treated PMN (Fig. 5). LDH release by vehicle-treated PMN was improved compared to the unwashed vehicle-treated PMN but continued to be significantly greater than buffer controls.

Superoxide anion production after washing vitamin E and vehicle from the reaction mixture are shown in Figure 6. Vitamin E-treated PMN continued to release similar amounts of superoxide anion at 3.5 mg/dl (359 ± 55 nM/dl) compared to buffer controls (359 ± 55 nM/dl). At 5.0 and 10.0 mg/dl vitamin E,

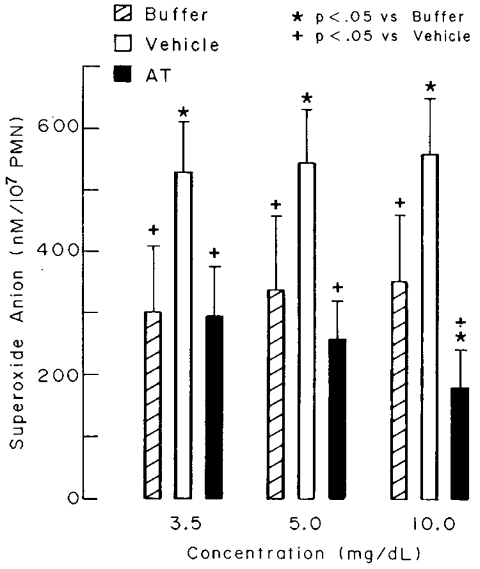

Fig. 3. Superoxide anion production—vitamin E dose-response with simultaneous exposure to vitamin E (AT) or vehicle and PMA. The amount of superoxide anion produced decreases with increasing concentrations of AT and becomes significantly lower at 10 mg/dL AT compared to buffer controls. Both buffer- and AT-treated PMN produce significantly less superoxide anion than vehicle-treated cells. The *bars* depict the mean ± 1 SD from five experiments.

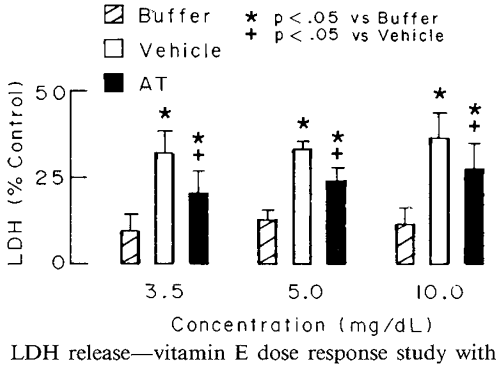

Fig. 4. LDH release—vitamin E dose response study with simultaneous exposure to vitamin E (AT) or vehicle and PMA. LDH release is significantly increased in both AT- and vehicle-treated PMN (all concentrations) compared to buffer controls ($p < 0.05$). AT-treated cells release significantly less LDH then vehicle-treated PMN. The *bars* depict mean ± 1 SD from five experiments.

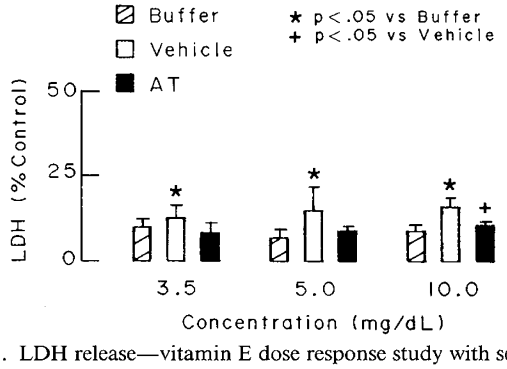

Fig. 5. LDH release—vitamin E dose response study with sequential exposure to vitamin E (AT) or vehicle and PMA. LDH release is similar for buffer- and AT-treated cells except at 10.0 mg/dl AT (small but significant difference). LDH release by vehicle-treated cells is significantly greater than by buffer-treated cells at 3.5, 5.0, and 10.0 mg/dl of vehicle. The *bars* depict the mean ± 1 SD from five experiments.

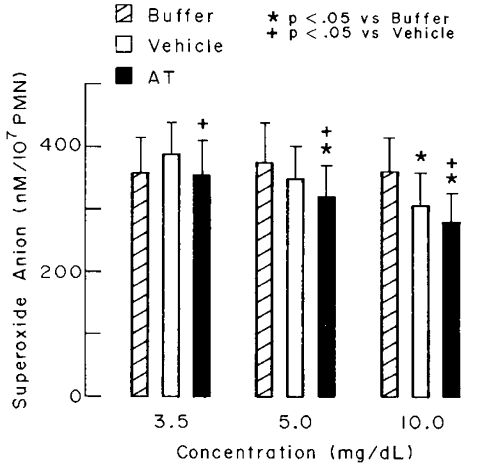

Fig. 6. Superoxide anion production—vitamin E dose response study with sequential exposure to vitamin E (AT) or vehicle and PMA. Superoxide anion production is significantly lower by AT-treated PMN than buffer controls at AT concentrations of 5.0 and 10.0 mg/dl. At 10.0 mg/dl of vehicle, superoxide anion production is significantly lower than buffer controls and significantly greater than AT-treated PMN. The *bars* depict the mean ± 1 SD from five experiments.

however, significantly less superoxide anion was produced (321 ± 49 and 278 ± 48 nM/10^7 PMN, respectively). Compared to vehicle-treated PMN, vitamin E-treated PMN produced less superoxide anion at all concentrations studied (Fig. 6). At a vehicle concentration equivalent to that in which 10 mg/dl of vitamin E is suspended, superoxide anion production was also significantly less than superoxide anion production from buffer-treated PMN.

DISCUSSION

Superoxide anion is an oxygen radical important for bacterial killing by polymorphonuclear leukocytes (15, 17). In the presence of pharmacologic levels of vitamin E, the ability of PMN to migrate toward a chemoattractant (9), destroy bacteria (10), and produce oxygen radicals (11, 12) is diminished. Baehner *et al.* (11) supplemented the diet of human volunteers with megadoses of vitamin E to achieve vitamin E levels twice normal; diminished bactericidal activity was found and was partly due to decreased hydrogen peroxide release. Shigeoka *et al.* (12) incubated polymorphonuclear leukocytes from two stressed neonates and adult controls with pharmacologic concentrations of vitamin E. A significant decrease in chemiluminescence (measure of total oxygen radical generation) was found and was most significant in leukocytes from stressed neonates.

Baehner *et al.* (11) did not show decreased superoxide anion production by PMN obtained from adult volunteers whose diets were supplemented with vitamin E. Our results may differ because we studied PMN exposed to 3.5–10 mg/dl vitamin E whereas Baehner *et al.* (11) studied PMN exposed to 1.77–1.85 mg/dl vitamin E. In addition, variation in experimental methods may have contributed to the differences found.

We treated polymorphonuclear leukocytes with levels of the vitamin E preparation reported by Johnson *et al.* (8) to be associated with a significant increase in infections in vitamin E-treated low birth weight infants. Our results demonstrate that vitamin E-treated PMN produce significantly less superoxide anion compared to vehicle-treated cells at 5 and 10 min of PMA exposure. Our dose-response studies demonstrated that at levels of vitamin E considered upper normal (3.5 mg/dl), superoxide

anion production is similar to buffer-treated controls (Fig. 3 and 6). However, superoxide anion production was significantly decreased at 5.0 and 10.0 mg/dl vitamin E compared to buffer-treated control cells (Fig. 6). Because results were unchanged after washing the detergents in the vehicle from the reaction mixture, we propose that this effect is due to the vitamin E incorporated into the cell membrane.

As adult and neonatal leukocytes have similar superoxide anion generating activity (18), one may assume that superoxide anion production by neonatal leukocytes will also be depressed by pharmacologic levels of vitamin E. Although the absolute decrease in superoxide anion production is only 11% at 5.0 mg/dl vitamin E and 23% at 10.0 mg/dl, in the presence of a limited neonatal host defense response (12, 19, 20), the neonate may be at heightened risk for infection. The mechanism of this vitamin E effect is unclear. As vitamin E is closely bound to arachidonate in the proximity of membrane-bound proteins (21), it is reasonable to hypothesize that pharmacologic concentrations of vitamin E may alter the membrane-bound components of the superoxide anion generating system thereby affecting its function.

The vehicle for vitamin E results in markedly increased superoxide anion production by PMN stimulated by PMA. The effect is mitigated by washing the vehicle from the reaction mixture before PMA exposure. Because LDH and superoxide anion production are both significantly increased when vehicle and PMA are concurrently present, it may be that some component of the vehicle primes the cell such that PMA more readily activates the enzymatic pathway responsible for superoxide anion production.

The protective function of vitamin E against oxidative attack and vehicle is demonstrated in Figures 2 and 4. In the presence of high concentrations of superoxide anion after stimulation of leukocytes exposed to vehicle alone, LDH release was markedly elevated. However, with the addition of vitamin E significantly less LDH release occurred (Fig. 4). This suggests that vitamin E protects the cell membrane of PMN from the action of the vehicle and local oxygen radicals.

In summary, *in vitro* exposure of PMN to pharmacologic levels of vitamin E is associated with significantly decreased superoxide anion production. These results may help explain the previous report of diminished hydrogen peroxide release with excess vitamin E because hydrogen peroxide is derived intracellularly from superoxide anion (11, 18). This finding may also explain, in part, the observation of Johnson *et al.* (8) that low birth weight infants maintained at pharmacologic levels of vitamin E are at increased risk for developing sepsis and necrotizing enterocolitis.

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