Consequences of Vitamin-E Deficiency on the Phagocytic and Oxidative Functions of the Rat Polymorphonuclear Leukocyte

By Richard E. Harris, Laurence A. Boxer, and Robert L. Baehner

Vitamin E serves an important antioxidant role in cellular membranes by blocking the peroxidation of polyunsaturated fatty acid (PUFA) constituents. Clinically, vitamin-E deficiency in premature infants may manifest itself as edema and hemolytic anemia. Thus, red blood cells lacking this vitamin are vulnerable to hemolysis by hydrogen peroxide in vitro. Premature infants, upon exposure to high concentrations of oxygen, are also vulnerable to developing retrolental fibroplasia and bronchopulmonary dysplasia. Both of these disorders can be ameliorated by administration of vitamin E.

Another model of oxidant-induced injury to cells that can be attenuated by vitamin E is the autooxidative damage to normal human polymorphonuclear leukocytes (PMN), which occurs during phagocytic challenge. Placement of PMN in an anaerobic environment, or repletion of PMN with vitamin E, enhances the ability of the cells to respond to chemotactic stimuli or ingest particles. The vitamin-E replete PMN release less hydrogen peroxide (H$_2$O$_2$) but not superoxide (O$_2^-$) than normal cells during phagocytosis, which is consistent with the notion that H$_2$O$_2$ initiates the peroxidative damage to the PMN. To further dramatize the important role of vitamin E in phagocytic cell function, we have chosen to study the consequences of vitamin-E deficiency on rat PMN.

MATERIALS AND METHODS

Vitamin-E-Deficient and Control Diets

Male weanling Sprague-Dawley rats were fed normal rat chow containing 100 ppm of alpha-tocopherol or the same diet but totally deficient in alpha-tocopherol (ICN Pharmaceuticals, Inc., Cleveland, Ohio) and balanced for elemental iron and polyunsaturated fatty acids (PUFA). On the respective diets, the weight gain in the two groups was equivalent over the duration of the study.

Preparation of Cells for Functional Studies

The rats were injected intraperitoneally with 30cc of 1.2% casein in normal saline (NS) and then sacrificed 40 hr later. The abdomen was lavaged with NS containing 10 U/ml of preservative-free sodium heparin. The peritoneal exudate was placed over a Ficoll-Hypaque gradient and centrifuged 35 min at 4°C, which yielded a pellet containing 95% PMN. The total number of PMN obtained from each lavage was quantified. The PMN fraction was washed 3 times in NS and resuspended in Kreb’s Ringer phosphate buffer (KRP).

Biological Studies

Total serum alpha-tocopherol levels were quantified by the method of Quaife et al. The extent of peroxidation of polyunsaturated membrane lipids of PMN was assessed by quantifying the formation of malonaldehyde using the thiobarbituric acid reagent according to the method of Stossel et al.

Functional Studies

Those functional studies that depend in part on intact functional microtubules include adherence, degranulation, and lectin-induced
Adherence of PMN to nylon columns was performed according to a previously described method. Degranulation was determined by exposing $5 \times 10^6$ PMN to opsonized zymosan at a final particle-to-cell concentration of 30:1 for 15 min at 37°C with or without a 5-min preincubation of 5 μg of cytochalasin B to render them secretory. Following incubation, the reaction mixtures were centrifuged at 1000 g for 10 min at 4°C. The cell-free supernatants were removed and assayed for beta-glucuronidase and lactic acid dehydrogenase. Total enzyme activity was measured in simultaneously run duplicate reaction mixtures to which had been added 0.2% Triton X-100 (Rohn and Hoss, Co., Philadelphia, Pa.) to release all of the available enzyme. Concanavalin-A-induced surface membrane capping was performed by the method of Oliver. A quantity of 10^6 PMN in 1 ml of KRP were incubated with 10 μg of fluorescein isothiocynate-conjugated concanavalin A (FITC-Con-A) for 5 min at 37°C. The cells were fixed with the addition of 1% of 4% formalin to the cell suspension and kept at room temperature for 10 min, then pelleted and placed on a glass slide with a cover slip and examined under the fluorescence microscope.

Those PMN functions that depend in part on the integrity of the plasma membrane, random motility, and chemotaxis were assayed by observing the ability of PMN to migrate through 5-μm Nucleopore filters employing a modified Boyden chamber (Neuroprobe chambers, Bethesda, Md.) as described by Hill et al. PMN (2.5 × 10^6) were placed in the upper compartment and the chemotacticant (bacterial filtrate from cultures of E. coli) in the lower compartment. The incubation was carried out in a 10% CO2-air, 37°C incubator for 90 min. The bottom side of the filters were then stained with Wright-Giesma and five 100× oil immersion fields were counted.

Ingestion of both C3b and IgG opsonized particles was determined by the method of Stossel et al. using E. coli lipopolysaccharide (LPS) coated or albumin-coated paraffin oil droplets containing oil red O (ORO), which had been opsonized with normal rat serum at 37°C for 25 min or with bovine serum albumin-rabbit IgG anti-BSA, respectively, to allow deposition of C3b or IgG on the surface of the droplets, respectively. In some studies, the samples were preincubated for 2 hr under a nitrogen environment before ingestion was initiated by the addition of the appropriately prepared ORO paraffin oil droplets. Ingestion of bovine serum albumin (BSA) coated oil red O paraffin oil droplets, which had not been opsonized with anti-BSA, was assayed as a measure of nonspecifically mediated ingestion. Ingestion of 3H-labeled Staphylococcus aureus 502A, opsonized with normal rat serum, was determined at bacteria-to-cell ratios of 10:1 and 4:1.

The killing of Staphylococcus aureus 502A was assayed by the previously described method of Quie et al. PMN (10^6) were incubated at 37°C for 100 min with S. aureus 502A at a cell-to-bacteria ratio of 1:2 and 1:8.

Oxidation-Reduction Responses of PMN

The extracellular release of hydrogen peroxide during phagocytosis of C3b-opsonized zymosan particles at a ratio of approximately 60 particles/cell was quantified by the method of Root et al. by measuring the decrease in fluorescence intensity of scopoletin during its oxidation by horseradish peroxidase. Next, 2 × 10^6 PMN were incubated at 37°C for 5 min in KRP with 5 mM glucose and 1 mM sodium azide. The cells were pelleted (250 g for 10 min). Then, to 1 ml of the supernatant was added 0.4 ml of 50 μM scopoletin (Sigma) in KRP and 0.4 ml of horseradish peroxidase (1 mg/ml). The decreased fluorescence of the mixture was quantitated in a spectrophotometer as compared to a blank containing KRP in the place of the cellular supernatant at an excitation wavelength of 350 nm and an incidence reading of 460 nm. The quantity of H2O2 generated was calculated from a graph generated from dilutions of an H2O2 standard solution prepared daily based on an extinction coefficient of 81 cm M^-1 at an optical density (OD) of 230 nm. Oxygen consumption during phagocytosis was quantified using a Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) equipped with a continuous recorder, as previously described. Oposized zymosan particles were employed instead of latex, since it has been our experience that rat PMN ingest latex particles very slowly. Reduced growth serum hormone (GSH) levels at rest and following 5 min of phagocytic challenge with opsonized zymosan were determined by the method of Burchill et al. Phagocytosis of opsonized zymosan was terminated at the end of 5 min by brief centrifugation of the cell suspension in a Beckman microcentrifuge, followed by rapid withdrawal of the supernatant medium into separate tubes on ice. The cell pellet was then fixed with gluteraldehyde at room temperature and processed for electron microscopy or stained with Wright’s stain and scored for phagocytosis by determining the percentage of 100 PMN with ingested particles using light microscopy.

Repletion Studies

To study whether the defect in PMN phagocytosis and chemotaxis could be corrected by in vivo repletion of the vitamin-E-deficient rats, the appropriate studies were repeated 3, 5, and 18 hr after repletion of the rats in vivo with 10 U of intramuscularly administered parenteral vitamin E (Hoffmann-La Roche Inc., Nutley, N.J.).

Statistical Analysis

Studies were run in quadruplicate, and each study was performed on individual animals. The results obtained from the vitamin-E-deficient PMN were compared to the control PMN utilizing the Student’s t test for the significance between two sample means.

RESULTS

Biochemical Studies

The serum vitamin-E level of the rats fed the vitamin-E-deficient diet for 2 mo was 0.06 ± 0.01 mg/dl compared to the control levels of 1.17 ± 0.06 mg/dl (only 5.1% of normal). The vitamin-E-deficient PMN had almost a twofold increase in peroxidized membrane polyunsaturated lipids as determined by the formation of malonaldehyde (252 ± 17 pM MDA/10^7 cells versus control of 138 ± 17 pM).

Functional Studies

Adherence of the vitamin-E-deficient PMN to a 70-mg nylon-fiber filter (57.5% ± 2.5%) was not significantly different from controls (55.0% ± 1.0%). Similarly, the release of beta-glucuronidase to the media following phagocytic challenge with opsonized zymosan was similar to controls (21.5% ± 1.5% compared to 21.0% ± 1.0% from control PMN). When phagocytosis was inhibited with cytochalasin B, the normal and deficient rat PMN discharged 89% ± 3%
and 92% ± 2% of their beta-glucuronidase, respectively. Under the latter circumstances, lactic acid dehydrogenase, a cytoplasmic enzyme, was present at a concentration of less than 2% in the extracellular medium, indicating that cell death had not occurred.

The percentage of PMN exhibiting spontaneous migration of FITC-Con-A membrane receptors into polar caps in vitamin-E-deficient PMN was similar to controls (9.2% ± 3.1%; controls 12.2% ± 2.3%). When the cells were treated with colchicine, an agent known to prevent the assembly of microtubules, both the vitamin-E-deficient and control PMN exhibited capping of 49% and 45%, respectively.

The recovery of peritoneal PMN in the vitamin-E-deficient rats was consistently lower than in controls (1.48 ± 0.40 x 10^8 PMN versus control 2.42 ± 0.36 x 10^8, p < 0.05). Chemotaxis in contrast to random motility of PMN was diminished in the vitamin-E-deficient rats (Table 1). In addition to impaired chemotaxis, ingestion of C3b- and IgG-coated paraffin oil droplets, as well as unopsonized albumin-coated droplets, was significantly diminished (p < 0.005) in the vitamin-E-deficient PMN (Table 2). To determine whether the impairment occurred antecedant to or at the time of the phagocytic oxidative burst, the paraffin oil ingestion studies were performed in an anaerobic environment (Table 2). The ingestion rate of the vitamin-E-deficient PMN remained depressed; whereas the ingestion rate of the replete PMN was potentiated.

Ingestion of ^14^C-labeled Staphylococcus aureus 502A was completely normal at time points 0, 1, 2, 5, 10, and 20 min as long as the bacteria-to-cell ratio was kept below 10:1. Killing of Staphylococcus aureus 502A was also normal again as long as the system was not stressed by keeping the bacteria:cell ratio less than 10:1 (data not shown). During phagocytic challenge, oxygen consumption and H_2O_2 release were found to be significantly increased in the vitamin-E-deficient PMN over control (Table 3). Uptake of opsonized zymosan by approximately 90% and 95% of the PMN obtained from both the vitamin-E-deficient and normal rates, respectively, occurred at 5 min as judged by light microscopy. On the average, each of the vitamin-E-deficient cells contained 3.9 particles, and the normal PMN contained 4.3 particles by transmission electron microscopy. Furthermore, reduced GSH levels in the resting state from the vitamin-E-depleted PMN were 650 ± 110 n mole/10^7 PMNs and were not significantly different compared to control values of 670 ± 40 n mole/10^7 PMNs, and following 5 min of phagocytic challenge, the GSH levels were 490 ± 100 n mole/10^7 PMN and were not significantly different compared to 530 ± 100 n mole/10^7 PMN in the respective populations. These results indicated that the pool of GSH was not substantially depressed in the vitamin-E-depleted PMN either at rest or following phagocytic challenge.

Repletion Experiments

When 10 mg of vitamin E were administered intramuscularly to the vitamin-E-deficient rats before harvesting the PMN, the paraffin oil ingestion rates

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### Table 1. Random Motility and Chemotaxis in Vitamin-E-Deficient and Control PMN

<table>
<thead>
<tr>
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<th>Random Motility* (no E. coli LPS)</th>
<th>Chemotaxis* (With E. coli LPS)</th>
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<tr>
<td>Normal</td>
<td>78 ± 5</td>
<td>161 ± 10</td>
</tr>
<tr>
<td>Vitamin-E-deficient</td>
<td>80 ± 3</td>
<td>105 ± 9</td>
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*The values represent the total number of cells observed in five 100x oil immersion fields when the chemotactrant was either absent (random motility) or present (chemotaxis). The values represent the mean ± SD of 5 separate experiments performed in quadruplicate.

### Table 2. Ingestion of ORO-Paraffin Oil Droplets by Vitamin-E-Deficient and Control PMN

<table>
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<tr>
<th></th>
<th>Controls</th>
<th>Vitamin-E-Deficient</th>
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<tbody>
<tr>
<td>C3b-receptor-mediated ingestion*</td>
<td>0.0465 ± 0.0045</td>
<td>0.0256 ± 0.0036</td>
</tr>
<tr>
<td>Aerobic</td>
<td>0.0582 ± 0.0042</td>
<td>0.0214 ± 0.0006</td>
</tr>
<tr>
<td>IgG-mediated ingestion†</td>
<td>0.0479 ± 0.0049</td>
<td>0.0262 ± 0.0015</td>
</tr>
<tr>
<td>Aerobic</td>
<td>0.0577 ± 0.0039</td>
<td>0.0243 ± 0.0007</td>
</tr>
<tr>
<td>Nonspecific mediated ingestion†</td>
<td>0.0301 ± 0.0029</td>
<td>0.0224 ± 0.0017</td>
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</table>

*All values are expressed in mg of ORO-paraffin oil/10^7 cells/min. These studies represent the mean ± SD of 10 separate experiments performed in quadruplicate. The results for each of the three droplets is significant at p < 0.0005.
†The LPS-coated paraffin oil droplets were opsonized with fresh rat serum.
‡The albumin-coated paraffin oil droplets were opsonized with anti-bovine albumin.

### Table 3. Oxidative Metabolism of Vitamin-E-Deficient and Control PMN

<table>
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<tr>
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<th>O_2 Utilization*</th>
<th>H_2O_2 Release†</th>
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<tbody>
<tr>
<td>Controls</td>
<td>0.714 ± 0.13</td>
<td>14.8 ± 0.8</td>
</tr>
<tr>
<td>Vitamin-E-deficient</td>
<td>0.889 ± 0.14</td>
<td>18.0 ± 1.2</td>
</tr>
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*The values represent the mean ± SD of 5 separate experiments performed in quadruplicate during the ingestion of opsonized zymosan.
†μmole O_2/10^7 cells/hr. p < 0.0005.
‡nmole H_2O_2/10^7 cells/10 min. p < 0.025.
remaind abnormal at 3 hr but were fully corrected by 5 hr (Table 4). In contrast, it took 18 hr of in vivo exposure to vitamin E to fully correct the chemotactic defect (data not shown), which correlated with normalization of the amount of polyunsaturated lipids in the PMN (145 ± 11 pmol MDA/10^7 cells).

**DISCUSSION**

Alpha-tocopherol, the most important form of vitamin E, is a fat-soluble membrane-associated antioxidant that chemically is a methyl substituted 6-hydroxy chroman derivative with a 16-carbon aliphatic side chain (Fig. 1). Its lipid solubility is accounted for by the fatty acid side chain, whereas its ability to trap lipid-free peroxide radicals resides in the phenolic group on the chroman ring. Vitamin E functions in cellular membranes by inhibiting the chain propagation phase of peroxidation of membrane-associated polyunsaturated fatty acids (PUFA). The consequences of vitamin-E deficiency should therefore be reflected by augmented membrane lipid peroxidation.

Such an event was evident by the increased formation of malonaldehyde (MDA) in the vitamin-E-deficient rat PMN. Malonaldehyde, a labile aldehyde, is the product of peroxidation of certain unsaturated fatty acids and is employed as an index of lipid peroxidation of polyunsaturated fatty acids.

Enhanced MDA formation is also observed in vitamin-E-deficient red cells. The vitamin-E-deficient red blood cells demonstrate increased hemolysis, which is associated with loss of unsaturated fatty acids from the membrane. Although fatty acid composition of the vitamin-E-deficient PMN was not determined in this study, the deficient PMN did have evidence of altered membrane properties.

The vitamin-E-deficient PMN were unable to respond normally to both chemotactic and phagocytic stimuli. Although the vitamin-E-deficient PMN responded more favorably to opsonized particles than unopsonized particles, the deficient PMN remained impaired in their overall ability to ingest particles normally. Sufficient oxidant damage to the membrane of the vitamin-E-deficient PMN had occurred such that placement of the cells in an anaerobic environment failed to improve chemotaxis and ingestion rates, respectively. In contrast, the normal rat PMN demonstrated enhanced phagocytic function by these manipulations, as demonstrated previously. These observations suggest that oxidant-inflicted damage to the vitamin-E-deficient PMN occurred antecedent to the phagocytic event; whereas, autooxidative damage to vitamin-E replete PMN occurs in tempo with the phagocytic event.

The phagocytic and chemotactic defects, however, are rapidly corrected by in vivo repletion of the deficient rats with parenteral vitamin E. The phagocytic defects are still present 3 hr after repletion but corrected by 5 hr, and chemotaxis is normalized within 18 hr and PMN polyunsaturated lipids are restored to normal. This interval likely represents the time required for absorption and incorporation of vitamin E into PMN membranes of exudate cells or indirectly into membranes of newly forming cells in the bone marrow which were then released into exudates. The phagocytic defect was evident when the cells were exposed to a saturating concentration of particles. In contrast, the vitamin-E-deficient PMN were able to normally ingest and kill limited numbers of bacteria.

Further evidence for alterations in the membrane milieu in the vitamin-E-deficient PMN is suggested by the alterations in the pyridine nucleotide-dependent oxidase activity of the PMN during phagocytic challenge. With phagocytic stimulation, the vitamin-E-deficient PMN, although compromised in their ability to ingest particles, manifested enhanced O2 consumption and H2O2 release beyond that observed for the normal cells. It is possible that the altered membrane environment in the vitamin-E-deficient PMN allows for conformational changes in the pyridine-dependent oxidase activity.
oxidase enzymes presumed to be localized to the membrane or in enhanced avidity of the enzyme for substrate or divalent cation cofactors, which in turn would permit enhanced activation.

There was no evidence for injury to the vulnerable sulfhydryl-bearing microtubules. The microtubule-dependent functions of lysosomal secretion and FITC-Con-A capping as well as adherence proceeded unimpaired in the depleted PMN. Even during phagocytic challenge, GSH levels remained substantially protected the vulnerable sulfhydryl-bearing microtubules from oxidative damage. We inferred that microfilament integrity also was not disturbed in the deficient PMN because the deficient PMN generated polar FITC-Con-A caps in response to colchicine, which occurs only in the presence of intact microfilaments. Furthermore, the extracellular release of beta-glucuronidase by the vitamin-E-deficient PMN could be enhanced to the same extent as by control PMN after treatment with the microfilament disrupting agent, cytochalasin B. Thus, the oxidant damage in the vitamin-E-deficient PMN was selective for those functions of the membrane involved in triggering PMN phagocytic and oxidative responses.

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ABNORMAL FUNCTIONS OF VITAMIN E DEFICIENT RAT PMN


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