Vitamin E—A Selective Inhibitor of the NADPH Oxidoreductase Enzyme System in Human Granulocytes

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The cellular sites of H_2O_2 formation in phagocytizing granulocytes have been identified with cerium chloride. A precipitate was visible in phagosomes and on plasma membranes from intact normal cells in the presence of either 0.71 mM NADH or NADPH. X-ray microanalysis permitted identification of cerium deposition within the phagosomes even in the absence of reduced pyridine nucleotides. Catalase ablated the formation of the reaction product. Intact granulocytes obtained from subjects receiving 1600 units of vitamin E daily for 2 weeks exhibited reaction product in the presence of NADH but not NADPH. Intact cells from subjects treated with vitamin E demonstrated diminished numbers of phagocytic vesicles containing reaction product. During phagocytosis the granulocytes treated with vitamin E consumed oxygen but exhibited significantly reduced rates of hydrogen-peroxide–dependent glucose-1-^{14}C oxidation to $^{14}CO_2$. Isolated phagocytic vesicles obtained from granulocytes after ingestion of opsonized lipopolysaccharide–paraffin oil droplets contained reaction product when exposed to 0.71 mM NADPH. No reaction product was evident at 0.71 mM NADH but was evident at 2.0 mM NADH. Isolated phagocytic vesicles from the granulocytes of subjects receiving vitamin E exhibited reaction product only in the presence of NADH. These observations suggest that vitamin E interferes with the electron transport chain apparently required for the oxidation of NADPH to form H_2O_2 in the phagocytizing granulocyte. (Am J Pathol 1983, 112:287–293)

EOSINOPHIL (EOs) and polymorphonuclear neutrophilic granulocytes (PMNs) exhibit a phagocytic oxidative burst. Reaction products associated with this burst are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical.[1] The major sources of electrons and hydrogen ions for the products are derived from reduced pyridine nucleotides, either NADH or NADPH. In turn, the oxidative products are toxic to bacteria, fungi, the surrounding tissues and to the PMNs themselves.[1] By interfering with the release of the oxidative products to the extracellular media, antioxidants can modify the toxic effects of the oxidative products on the function of the PMNs.[2] Alpha-tocopheral (vitamin E) will cause the human PMNs to release substantially less H_2O_2 to the extracellular media and to phagocytize at faster rates.[2] The mechanism by which vitamin E modifies granulocyte function is not entirely clear. We previously suggested that vitamin E selectively scavenges hydrogen peroxide in preference to superoxide.[2] The enzymatic basis for these reduced oxygen by-products is thought to arise from a series of coupled electron transport reactions involving a multienzyme complex system.[3,4] Part of these components have been suggested to reside within the plasma membrane.[5] Since vitamin E is intercalated readily into membranes,[6] we conjectured that the impairment in hydrogen peroxide release might be directly affected by the presence of vitamin E. We employed ultrastructural analysis, which allowed us

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to clarify the mechanisms by which vitamin E modifies the respiratory burst of PMNs.

Materials and Methods

PMNs were obtained from the peripheral blood of normal human controls by a previously described method.[2] PMNs were isolated by centrifugation of the blood over Ficoll-Hypaque followed by lysis of the erythrocytes in the pellet with isotonic ammonium chloride as described.[7] The cells were washed and suspended in Krebs-Ringer phosphate (KRP), pH 7.4. The PMN suspension was approximately 97% pure with 2-3% contamination with EOs. Three volunteers were studied before and during vitamin E ingestion (400 IU vitamin E capsules 4 times a day for 14 days). In all cases, informed consent was obtained in accordance with the standards of the human investigational committee at this institution. The total level of serum α-tocopherol was determined by the microassay of Quaife and co-workers.[8]

Metabolic Studies

To assess an intracellular metabolic reaction propelled by H_2O_2, the activity of the hexose monophosphate shunt was studied in PMNs at rest and during phagocytosis of opsonized zymosan particles over 30 minutes by a previously described method.[9] To assess the role of H_2O_2 to stimulate the hexose monophosphate shunt, 0.1 mM xanthine and 0.25 units/ml xanthine oxidase with or without 1500 units of catalase was added to control resting PMNs. The release of hydrogen peroxide from control PMNs phagocytizing opsonized zymosan in the presence of 1 mM cerium chloride or 0.71 mM NADPH was also quantified by the method of Root et al.[10] To confirm that vitamin E cells were capable of utilizing oxygen, control and vitamin E PMNs were incubated with and without 0.3 mg/ml or 1.0 mg/ml opsonized zymosan, and the rate of oxygen consumption was measured by our previously described method.[11]

Electron-Microscopic Studies

Whole Cells

To explore the ultrastructural site of the reduced-pyridine-nucleotide-dependent oxidase in PMNs and EOs before and after exposure to antioxidants, a cytochemical technique for the localization of H_2O_2 production in unfixed, phagocytizing PMNs developed by Briggs and co-workers was modified for this study.[12] Purified PMNs were suspended in autologous serum at a concentration of 1.5×10^7 cells/ml and allowed to adhere to 35-mm Petri dishes for 30 minutes at 37 C. Opsonized zymosan particles at a ratio of 20 particles/cell were added to the adhering PMNs and incubated with the cells at 37 C for 10 minutes. In some experiments the reaction was carried out in the presence of 1500 units of catalase. The supernate was then discarded, and 0.1 M Tris-maleate buffer, pH 7.6, containing 7% sucrose, 1 mM 3-amino-1,2,4,-triazole (AT) (Aldrich Chemical Co., Inc., Milwaukee, Wisc) was added for 10 minutes to inhibit granule myeloperoxidase. The supernate was decanted, and the final incubation medium consisting of 0.1 M Tris-maleate, pH 7.6, with 7% sucrose, 10 mM AT, 1 mM CeC13 with or without 0.71 mM NADH or NADPH (Sigma Chemical Co., St. Louis, Mo) was added for 20 minutes at 37 C. The final incubation medium was prepared within 10 minutes of use.

After incubation, the PMNs and EOs were washed briefly in 0.1 M Tris-maleate buffer with 7% sucrose at 25 C. The cells were then removed from the Petri dishes with a rubber policeman and suspended in the 0.1 M Tris-maleate buffer with 7% sucrose. The cells were then fixed in 3% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for one hour and washed with 0.1 M cacodylate buffer, pH 7.4, containing 7% sucrose at room temperature for 10 minutes. The cells were then postfixed in 1% osmium tetroxide in 0.1 M cacodylate, pH 7.4, containing 7% sucrose for additional 60 minutes at room temperature. The cells were then stained *en bloc* in 0.5% aqueous uranyl acetate overnight at 4 C, dehydrated in graded series of alcohol, and embedded in Spurr resin.[13] Thin sections were prepared on a Sorvall MT-2 ultramicrotome equipped with a diamond knife. Sections were routinely poststained with uranyl acetate and lead citrate and examined on a Philips 400 electron microscope at 80 kv. The electron micrographs were processed by an unbiased observer. The presence of cerium was verified by the use of X-ray microanalysis.

Phagocytic Vesicles

Phagocytic vesicles were isolated according to the procedure of Stossel et al.[14] PMNs were incubated with opsonized lipopolysaccharide-coated paraffin oil red O particles for 15 minutes at 37 C. Phagocytosis was halted by the addition of ice-cold KRP. The cells were pelleted and washed three times in KRP for the removal of excess oil red O particles. After the final centrifugation, cells were suspended in 0.34 M sucrose containing 1 mM potassium phosphate,

pH 7.0 (buffered sucrose). After the addition of 500 units of heparin to a 3-ml cell suspension to prevent subcellular particle aggregation, the cell membranes were disrupted in a chilled Thomas tight-fitting machine-driven homogenizer. Following 200 strokes over a 10-minute period, complete disruption of membranes was verified by phase-contrast microscopy.

The PMN homogenate was layered over an equal volume of 0.40 M buffered sucrose. In turn, this was overlaid with an equal volume of 0.25 M buffered sucrose and centrifuged at 100,000*g* for 1 hour at 4 C.

The resulting supernatant containing the phagocytic vesicles was decanted and filtered through a Unipore filter (Biorad Laboratories, Richmond, Calif) with a pore size of 0.1 μ. The vesicles on the filter were treated with an identical reaction mixture employed in the whole-cell preparation. In addition, some vesicles were reacted with 2.0 mM NADH. The vesicles were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4 C. After three buffer washes, the filters were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 hour at room temperature. The filters were then treated with a thin coat of 5% agar to prevent vesicle loss during dehydration and embedding procedures. The vesicles and the filter were dehydrated in ethanol and embedded in Spurr resin.

The vesicles were viewed on a Philips 400 electron microscope equipped with a goniometer stage and an EDAX 9100 energy dispersive X-ray microanalysis system. Twenty vesicles for each variable were studied for the presence of the cerium precipitate within the vesicle. The probe was positioned so that the entire contents of the vesicle could be examined by adjusting the diameter of the beam. Each vesicle was evaluated for 200 live seconds at 80 kev. The background continuum was subtracted from the X-ray spectra, and the integral counts for cerium L-alpha and L-beta spectral peaks were obtained. The statistical significance of the cerium peaks was verified with the use of the equation described by Roomans.[15] The data were subjected to nonparametric statistical analysis.

Results

Metabolic Studies

Following the administration of vitamin E for 14 days, the α-tocopheral level in the plasma rose from 1.12 ± 0.16 to 1.82 ± 0.04 mg/dl. Under such conditions, previous studies indicated that vitamin E administered *in vivo* enhanced the phagocytic rate of PMNs and increased O_2^- release while attenuating the release of H_2O_2 to the extracellular medium.[2] As indicated in Table 1, glucose-1-^{14}C oxidation rates were significantly decreased in phagocytizing PMNs from volunteers receiving vitamin E. On the other hand, oxygen consumption of PMNs stimulated with opsonized zymosan occurred in subjects receiving vitamin E. Oxygen consumption reflects an algebraic sum of oxygen used and oxygen released. These studies imply that vitamin E can interfere with H_2O_2 produced internally during the respiratory burst of the granulocyte. The stimulated rate of glucose oxidation achieved by incubation of PMNs with the H_2O_2 generating system xanthine-xanthine oxidase was completely inhibited by 1500 units of catalase, implicating the role of H_2O_2 as stimulator of glucose oxidation (data not shown). The release of H_2O_2 from normal PMNs phagocytizing opsonized zymosan in the presence of cerium chloride was significantly attenuated (10.72 ± 0.12 versus 8.44 ± 0.12 nmol H_2O_2/min/10^7 PMNs; $P < 0.01$). These studies directly implicate the utilization of H_2O_2 in the formation of cerium reaction product.

Electron-Microscopic Studies

Whole Cells

Following a 10-minute challenge with opsonized zymosan particles, both EOs and PMNs exhibited electron-dense reaction product primarily in the phagosome and to a much lesser extent on the cell plasma membrane (Figure 1A and B and Table 2). At sites of granule fusion with the phagosome, reaction product was occasionally observed in association with the granule contents. Channels connecting completely closed phagocytic vacuoles with the surface were typically lined with the reaction product. Ap-

Table 1—Effect of Vitamin E on the Respiratory Burst Activity in PMNs During Phagocytosis

Treatment	$^{14}CO_2$ from glucose-1-^{14}C nmol/min/10^7 cells		Oxygen consumption nmol/min/10^7 cells	
Control	99.6 ± 1.4	$P < 0.01$	62 ± 8	NS
Vitamin E	37.6 ± 0.3		67 ± 4	

Values are means ± SD of three separate experiments. The resting oxidation activities did not differ.

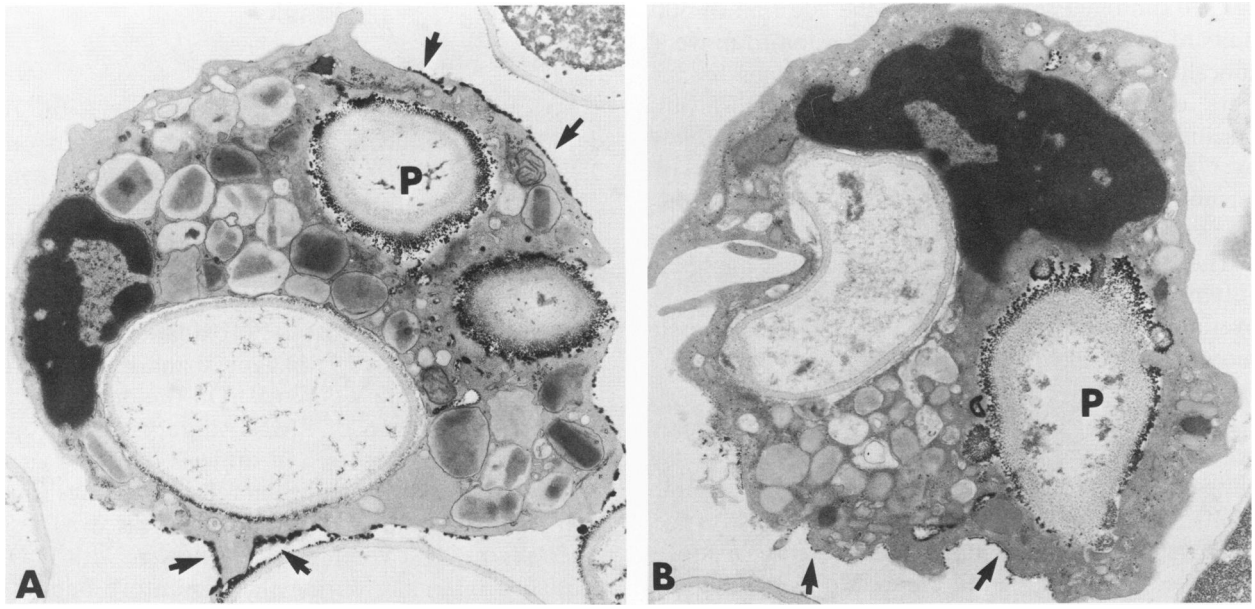

Figure 1—(A) The distribution of reaction product in EOs and PMNs (B) occurred within phagolysosome (*P*) and on the cell membranes (*arrows*) after incubation with 0.71 mM NADPH. The same distribution of reaction product could be found in cells incubated with 0.71 mM NADH. (×15,000) (With a photographic reduction of 8%)

proximately 60% of PMNs and EOs containing zymosan exhibited visible reaction product in the presence of one or the other of the reduced pyridine nucleotides (Figure 2A and B). X-ray microanalysis was required to demonstrate the presence of cerium precipitate in the absence of exogenous reduced pyridine nucleotides (Table 2). The characteristic L-alpha and L-beta peaks occurred at 4.84 and 5.26 kev, respectively. With this technique, in the presence of added reduced pyridine nucleotides at 0.71 mM concentration, reaction product was identified in > 90% of the intact cell phagocytic vesicles. Resting PMNs and EOs rarely contained reaction product. Catalase in media devoid of AT prevented reaction product formation in phagosomes or on the cell membrane (Table 2). In contrast, PMNs obtained from subjects receiving vitamin E were able to generate reaction product only in the presence of 0.71 mM NADH (Table 2). In the absence of reduced pyridine nucleotide, catalase prevented the reaction product, whereas vitamin E attenuated this response.

Phagocytic Vesicles

X-ray microanalysis was used to determine objectively the presence of cerium in isolated phagocytic vesicles. In contrast to the results observed in the whole cells with zymosan particles, phagocytic vesicles isolated after incubation with opsonized lipopolysaccharide-coated oil red O particles displayed a more dispersed cerium reaction product in the presence of 0.71 mM NADPH or 2.0 mM NADH. Minimal reaction product occurred in the presence of 0.71 mM NADH (Table 3). Reaction product was not evident in the absence of exogenous reduced pyridine nucleotide or in the presence of added catalase (Table 3). The precipitate was distributed throughout the contents of the vesicle and not merely associated with the membrane.

Phagocytic vesicles prepared from subjects receiving vitamin E showed little to no reaction product in the presence of 0.71 mM NADH and NADPH, respectively. In contrast, when 2.0 mM NADH was

Table 2—X-Ray Microanalysis for the Presence of Cerium Precipitates in Phagocytic Vesicles of Whole Human Polymorphonuclear Leukocytes After Zymosan Ingestion

Substrate	NADH (0.71 mM)*	NADPH (0.71 mM)*	No additive*
Untreated	19	18	9
Vitamin E	18 NS	0 $P = 0.005$	5 $P = 0.05$
Catalase (1500 units)	3 $P = 0.005$	2 $P = 0.005$	2 $P = 0.02$

* Number of positive vesicles. Twenty vesicles were analyzed per variable. *P* values represent comparisons between values observed in the untreated and vitamin-E-treated subjects and the untreated with and without catalase.

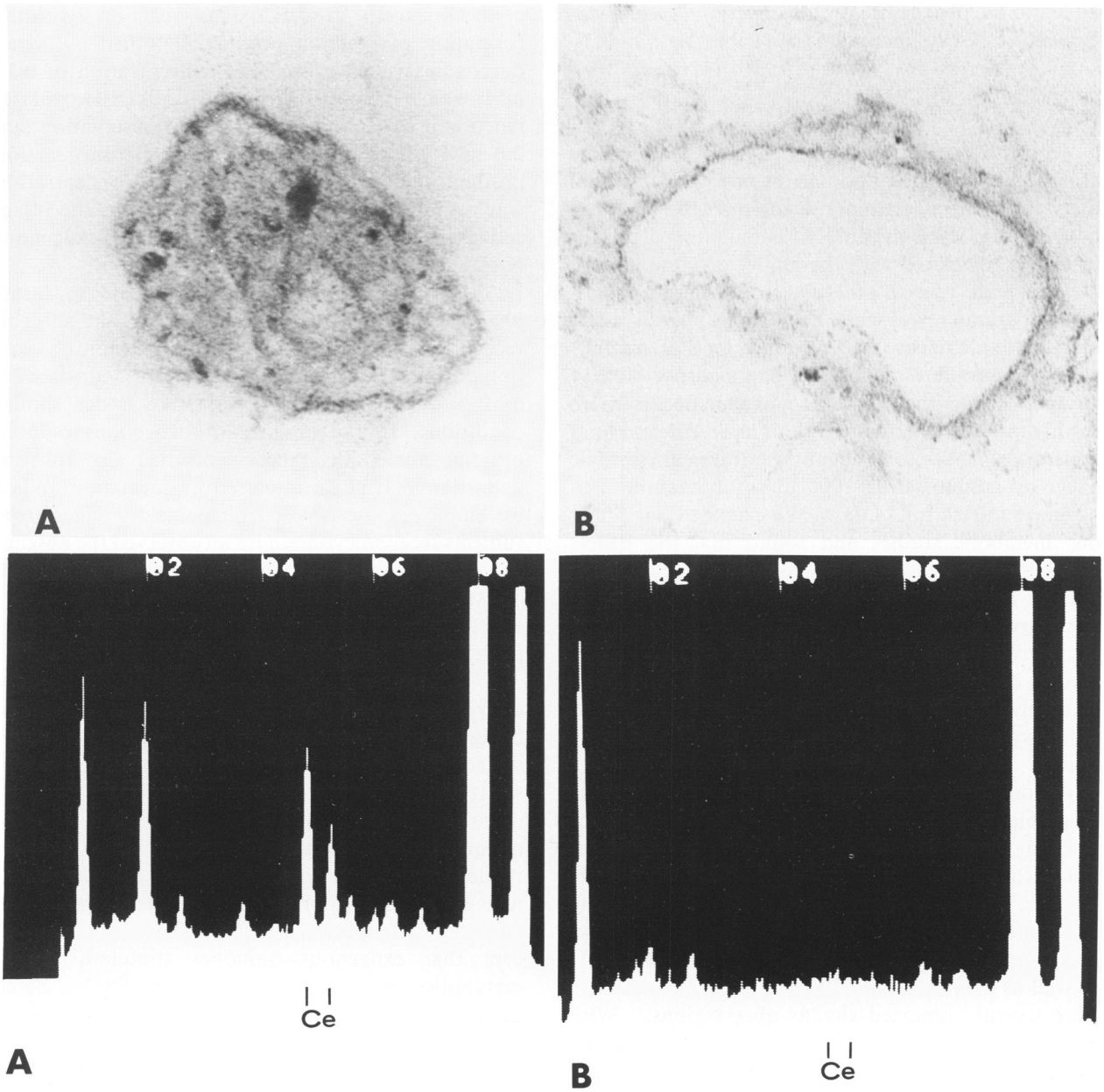

Figure 2—X-ray microanalysis provided an objective means of determining the presence of cerium in isolated phagocytic vesicles. In the spectra, the characteristic L-alpha and L-beta peaks for cerium can be seen at 4.84 and 5.26 kev, respectively. **A**—Cerium was detected in this normal vesicle after incubation with 0.71 mM NADPH. Reaction product could also be detected in vesicles after incubation with 2 mM NADH. **B**—Cerium was not detected in a vesicle when 0.71 mM NADH was used as substrate. Reaction product could not be observed in similar vesicles when catalase or vitamin E was used to block cerium precipitation when employing NADPH. Vertical scale, 1135 counts.

Table 3—X-Ray Microanalysis for the Presence of Cerium Precipitates in Isolated Phagocytic Vesicles From Human Polymorphonuclear Leukocytes After Paraffin Oil Ingestion

Substrate	NADH (0.71 mM)*	NADH (2.0 mM)*	NADPH (0.71 mM)*	No additive*
Untreated	3	16	18	0
Vitamin E	3 P = NS	14 P = NS	0 P = 0.005	0
Catalase (1500 units)	1 P = NS	0 P = 0.005	0 P = 0.005	0

* Number of positive vesicles. Twenty vesicles were analyzed per variable. P values represent comparisons between values observed in the untreated and vitamin-E-treated subjects and the untreated with and without catalase.

added to the phagocytic vesicle, reaction product was evident by X-ray microanalysis (Table 3).

Discussion

Very little attention has been given to vitamin E as a naturally occurring antioxidant in PMNs. Normal diets enriched in vitamin E lead to PMNs with enhanced phagocytic capability. The increased phagocytosis is correlated with the ability of vitamin E to attenuate the release of H_2O_2 from human phagocytizing granulocytes while having no apparent effect on superoxide release as determined by SOD-inhibitable cytochrome C reduction. The incorporation of vitamin E into the membrane bilayer occurs more readily *in vivo*, where it functions as an efficient lipid antioxidant.[16] We took advantage of this physiologic effect by administering 1600 units of vitamin E to normal subjects for 14 days. We extended our previous metabolic studies and found that the PMNs affected by vitamin E failed to significantly oxidize glucose-1-^{14}C to $^{14}CO_2$ in response to phagocytic challenge, but these same PMNs were able to consume oxygen as readily as control PMNs. These studies initially suggested to us that vitamin E was probably scavenging H_2O_2 as it was being generated from oxygen during the oxidase-catalyzed respiratory burst.[2]

We undertook an ultrastructural analysis to further elucidate the mechanism of the inhibition of H_2O_2 by vitamin E. We selected the cerium localization technique of Briggs and Karnovsky for NADH oxidase.[12] In our studies we observed cerium reaction product in both intact PMNs and eosinophilic granulocytes employing freshly prepared 0.71 mM NADH as well as 0.71 mM NADPH. Ohno and co-workers have recently reported similar observations.[17] With either reduced pyridine nucleotide, reaction product was prevented by the addition of the H_2O_2-scavenger catalase. Furthermore, utilizing a more sensitive technique of X-ray microanalysis, we were able to detect visible as well as nonvisible reaction product in the whole-cell phagocytic vesicles even in the absence of reduced pyridine nucleotides, which was again eliminated by exogenous catalase. Since the reaction product in the whole-cell preparations was largely confined to the phagocytic vesicle, we chose to isolate phagocytic vesicles to further define the optimal requirement for either reduced pyridine nucleotide. In media devoid of pyridine nucleotide, no reaction product was identified in isolated phagocytic vesicles. Unlike the intact cells, addition of 0.71 mM NADH failed to elicit a reaction product. However, increasing the amount of 2.0 mM, a concentration in excess of the Km of the NADH oxidase reaction led to the formation of reaction product identified by X-ray microanalysis. At a NADPH concentration of 0.71 mM, which exceeds the Km of the NADPH oxidase, reaction product also formed.[18] Our observation that 2.0 mM NADH was required to generate reaction product suggests that an equivalent concentration was achieved locally within the milieu of the intact cell even in the presence of 0.71 mM exogenous NADH.

Addition of NADPH as a substrate to intact phagocytizing granulocytes isolated from subjects receiving vitamin E blocked the formation of reaction product. Reaction product was formed when 2.0 mM NADH was used as substrate under similar conditions. In the absence of exogenous reduced pyridine nucleotide, reaction product also formed, and vitamin E again impaired its formation. When we employed the isolated phagocytic vesicles from subjects receiving vitamin E, we observed the absence of reaction product only in the presence of NADPH. These studies suggest that vitamin E has a selective effect in preventing the generation of H_2O_2 in the presence of NADPH.

A previous study implicated a flavin-containing dehydrogenase referred to as NAD(P)H oxidase as being responsible for the generation of H_2O_2 by stimulated human phagocytes.[19] However, a more recent study suggests that a nonmitochondrial B-type cytochrome may also be involved.[4] Others have identified a requirement for either a dichlorophenolindophenol reductase[20] and/or a ubiquinone in a multicomponent electron transport system[3] for the production of active oxygen species. Recent data support the concept that exogenous quinones stimulate oxygen metabolism in intact or in disrupted PMNs. Semiquinones are effective inhibitors of the respiratory burst.[3,21] Because vitamin E forms a semiquinone[22,23] following abstraction of hydrogen by another free radical, it is possible that the semiquinone structure of vitamin E (tocopherone) exerts its inhibitory effect by disrupting the flow of electrons from NADPH to O_2 in the multicomponent electron transport system. Vitamin E can also be oxidized to a hydroperoxide, consuming oxygen in the process.[16] Our data further suggest that NADH does not participate in the same manner to reduce oxygen to H_2O_2, because vitamin E failed to prevent the H_2O_2-dependent cerium reaction product in phagocytizing PMNs and in their isolated phagocytic vesicles. This hypothesis would account for the presence of some reaction product in the intact vitamin E cells lacking exogenous reduced pyridine nucleotides.

Further support for the formation of a semi-

quinone in the vitamin E PMNs stems from our prior observation that superoxide anion release was enhanced during phagocytosis in the face of impaired H_2O_2 release.[2] These observations can now be explained by the recent demonstration that semiquinone radicals are directly able to reduce cytochrome c, and this reaction is inhibitable by large concentrations of superoxide dismutase.[24] This would provide an artifact in the assay system falsely indicating normal release of "SOD-inhibitable" superoxide anion.

Our studies are most compatible with the multicomponent enzyme theory utilizing NADPH as the preferred substrate for the oxidase. Vitamin E appears to be a selective inhibitor of this complex and may provide a useful tool for further probing the individual components of the electron chain.

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