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Ascorbate and Phagocyte Function

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Scorbutic guinea pig neutrophils (PMN) were found to produce H_2O_2 and kill *Staphylococcus aureus* as well as control PMN, suggesting that ascorbate does not contribute significantly to phagocyte H_2O_2 production or bacterial killing. Total and reduced ascorbate contents of human PMN were observed to fall upon phagocytosis, whereas dehydroascorbate increased to a lesser extent. These observations are consistent with the view that ascorbate constitutes a functional part of the PMN's redox-active components and may thus function to protect cell constituents from denaturation by the oxidants produced during phagocytosis.

In a previous study (5), we found that human neutrophil leukocytes have impressive capacity for reducing dehydroascorbate and thus for regenerating their content of reduced ascorbate upon oxidation. This property of neutrophils (PMN), along with their relatively high ascorbate content, suggests that ascorbate may play an important role in PMN function. Phagocyte ascorbate might promote oxidative denaturation of bacterial components and thus potentiate bacterial killing, as proposed by Miller (18) and by Drath and Karnofsky (10). Ascorbate also might function to preserve cell integrity by inactivating free radicals and oxidants (8, 9, 26) produced during phagocytosis (2, 3, 14).

To determine whether physiological concentrations of ascorbate are critical for optimal phagocytosis and bacterial killing, we assayed H_2O_2 production and bactericidal activity in scorbutic guinea pig PMN. To examine the possibility that ascorbate is a redox-active component of oxidant-producing cells, we have measured changes in human PMN ascorbate contents during phagocytosis.

MATERIALS AND METHODS

Ascorbate contents. Ascorbate contents of tissues and cell preparations were measured by the method of Roe et al. (22). This method quantitatively distinguishes reduced ascorbate, dehydroascorbate, and diketogulonate from each other and from other organic compounds. Dehydroascorbate, the oxidized derivative of ascorbic acid, is reducible to ascorbate in most mammalian tissues. Diketogulonate, the hydrated derivative of dehydroascorbate, is not converted to dehydroascorbate in mammalian tissues.

Guinea pig experiments. Two-month-old guinea pigs weighing 325 to 375 g were divided into two groups. Both groups were fed for 18 days with an ascorbic acid-deficient diet (Nutritional Biochemicals Co., Cleveland, Ohio). Control animals were also fed by gavage 1 mg of ascorbate per g of body weight daily. Single experiments utilized pairs of animals, one scorbutic and one control. The order of harvest of control and scorbutic cells was alternated in successive experiments.

Guinea pig peritoneal PMN were harvested 12 h after intraperitoneal injection of 15 ml of 20% autoclaved sodium caseinate. Cells were suspended in calcium-free Krebs-Ringer phosphate buffer (KRP), pH 7.4. After 2 volumes of 0.87% ammonium chloride was added to lyse contaminating erythrocytes, leukocytes were collected by centrifugation for 10 min at 150 × *g*, washed twice in KRP, counted in a hemocytometer, and diluted with KRP to an approximate concentration of 100×10^6 phagocytes (PMN plus macrophages)/ml. Final suspensions contained 74 to 95% neutrophils, 3 to 25% macrophages, 0 to 8% lymphocytes, and 0 to 2% eosinophils.

H_2O_2 production was measured continuously as the rate of $^{14}CO_2$ production from [1-^{14}C]formate (1, 14) (New England Nuclear Corp., Boston, Mass.), using the gas flow-ionization chamber system of Davidson and Tanaka (7). This technique converts charge accumulated in an ionization chamber to a millivolt signal. Unstimulated reaction mixtures (1 ml) containing 20×10^6 to 40×10^6 leukocytes, 0.7 μmol of sodium formate including 0.86 μCi of [^{14}C]formate, and 5.6 μmol of glucose in KRP were incubated in 20-ml flat-bottomed glass vials in a Dubnoff shaking incubator (Precision Scientific Co., Chicago, Ill.) at 37 C, 80 oscillations/min. Phagocytosing signals were recorded after the addition of approximately 2×10^9 thrice-washed polystyrene latex spheres (0.81 μm in diameter; Bacto-Latex, Difco Laboratories, Detroit, Mich.) in 0.2 ml of KRP. The depth of the reaction mixtures was 2 mm and the surface area was 5 cm^2. Vials were gassed with 5% CO_2 in air, flowing at 73 ml/min. Nanomoles of formate oxidized were calculated from the millivolt signal, using factors derived from calibrations previously reported (5).

Bacterial killing by guinea pig peritoneal PMN was measured using a modification of the method of

Pincus and Klebanoff (21), within 2 to 4 h of cell collection. Assay mixtures (1 ml) contained 30×10^6 phagocytes, 5×10^6 colony-forming units of *Staphylococcus aureus* 502A (kindly supplied by G. Mandell), 0.1 ml of serum separated from blood obtained by cardiac puncture at the time of cell harvest, 10 μmol of glucose, and KRP. These were incubated at 37 C in stoppered siliconized glass tubes (12 by 75 mm), which were rotated on a model 150 Multi-Purpose Rotator (Scientific Industries, Inc.) at 24 rpm. At intervals noted in Fig. 1, 0.1-ml aliquants were removed, diluted in distilled water, vortexed heavily to disrupt PMN, and plated in duplicate in Trypticase soy broth containing 15% agar. Colonies were counted after 44 to 48 h of culture at 37 C.

Human experiments. Human PMN were separated from peripheral blood as previously described (5). These preparations contained 85% neutrophils, 5 to 15% monocytes, 0 to 8% lymphocytes, and 0 to 5% eosinophils.

Ascorbate contents of resting and phagocytosing PMN were measured by using the entire 1.5-ml reaction mixtures: 0.7×10^6 to 1.2×10^6 phagocytes, with or without approximately 2×10^9 latex particles, suspended in KRP containing 8.25 μmol of glucose. Samples were incubated in the shaking incubator at 80 oscillations/min at 37 C for the times indicated in the tables.

RESULTS

The ascorbate contents of scorbutic guinea pig peritoneal PMN, whole blood, liver, and kidney were about 15% of normal (Table 1). These values agree well with published data for comparably treated animals (20).

Scorbutic guinea pig leukocytes produced normal amounts of H_2O_2 during phagocytosis (Table 2). Four of thirteen scorbutic peritoneal exudates were grossly bloody. PMN in those samples were packed with ingested erythrocytes and exhibited high resting H_2O_2 production (mean, 1.69 nmol of formate oxidized/10 min per 10^6 PMN), which did not increase upon addition of latex particles. Giemsa-stained smears showed that less than 1% of these cells had ingested latex spheres. These findings were reproduced in PMN from a control guinea pig given isologous whole blood intraperitoneally 12 h before PMN harvest. Such bloody samples were excluded from this study.

Scorbutic PMN killed *S. aureus* as efficiently as did control guinea pig cells (Fig. 1). It can also be inferred from Fig. 1 that normal and scorbutic sera supported opsonization equally well, since phagocytosis-dependent bacterial killing did not vary significantly with serum source.

The total ascorbate content of phagocytosing human peripheral blood PMN decreased during the 60 min after phagocytosis and then remained stable (Table 3). Table 4 shows that total ascorbate decreased by an average of 12% in phagocytosing normal human PMN but did not change during phagocytosis in cells from two patients with chronic granulomatous disease. The decrease in total ascorbate content of phagocytosing normal cells was accompanied by a marked decrease in reduced ascorbate which was not accounted for by a moderate increase in dehydroascorbate. Only trace amounts of diketogulonate were detectable in both resting and phagocytosing samples.

DISCUSSION

The present study shows that PMN obtained from scorbutic guinea pigs produce H_2O_2 and kill *S. aureus* as well as do control cells, at least

TABLE 2. *H_2O_2 production by scorbutic and control guinea pig PMN without and with latex particles*

PMN	Formate oxidation[a]		Ratio (phagocytosing/unstimulated)
	Unstimulated	Phagocytosing	
Control[b]	0.532 ± 0.206	1.480 ± 0.552	2.8 ± 0.3
Scorbutic[c]	0.575 ± 0.294	1.401 ± 0.367	2.7 ± 0.7

[a] Expressed as nmol of formate oxidized/10 min per 10^6 PMN; mean ± standard deviation.
[b] $n = 10$.
[c] $n = 9$.

TABLE 1. *Total ascorbate content of scorbutic and control guinea pig tissues*

Tissue	Total ascorbate		Ratio (scorbutic/control)
	Control	Scorbutic	
Peritoneal PMN[a]	42.4 (34.8-51.0)	4.5 (3.2-5.5)	0.11
Blood[b]	4.9 ± 0.3	0.6 ± 0.17	0.12
Liver[c]	126.7 ± 23.8	18.8 ± 1.7	0.15
Kidney[c]	68.8 ± 10.7	10.8 ± 2.8	0.16

[a] $n = 3$ control, 3 scorbutic; expressed as nmol/10^6 cells; mean (range).
[b] $n = 6$ control, 6 scorbutic; expressed as nmol/0.1 ml; mean ± standard deviation.
[c] $n = 6$ control, 6 scorbutic; nmol/100 mg of tissue; mean ± standard deviation.

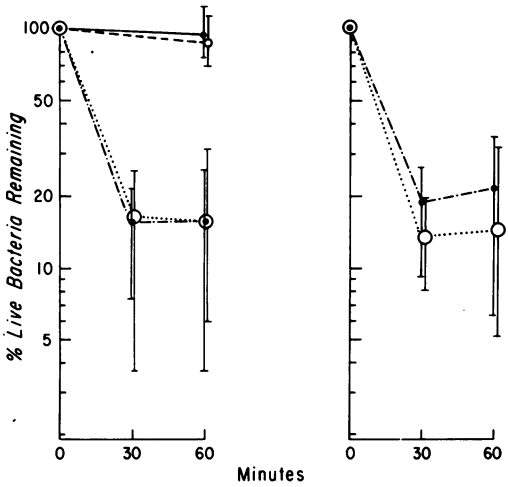

FIG. 1. *In vitro killing of S. aureus 502A by scorbutic and control guinea pig PMN. n = 3 for scorbutic PMN plus control serum; n = 4 for the other groups; mean values and ranges are indicated. Symbols for left-hand panel:* O- - -O, *Scorbutic serum;* ●-·-●, *control PMN plus serum;* ●——●, *control serum;* O....O, *scorbutic PMN + serum. Symbols for right-hand panel:* ●, *Control PMN plus scorbutic serum;* O, *scorbutic PMN plus control serum.*

briefly after phagocytosis. Since these functions depend on phagocytosis, our results argue against the conclusion of Nungester and Ames that phagocytosis is impaired in scorbutic guinea pig phagocytes (19). The discrepancy is best explained by the fact that the peritoneal exudates in the study of Nungester and Ames were hemorrhagic, as often occurs with advanced ascorbate deficiency. As we have demonstrated, erythrophagocytosis interferes with further particle ingestion and therefore with enhanced H_2O_2 production upon incubation with latex spheres. Experiments using bloody exudates were excluded from the present study.

The observations that H_2O_2 production and bacterial killing are unimpaired in scorbutic PMN imply that these activities are not sensitive to changes in ascorbate concentration over the range studied here. The 15% of normal ascorbate residual in scorbutic cells might suffice to support H_2O_2 production, since neutrophils possess efficient dehydroascorbate reducing activity (5). However, it would be unusual for a biological reaction to be insensitive to a decrease in substrate concentration to less than one-fifth of the physiological level. Therefore, we doubt that ascorbate participates directly in phagocyte H_2O_2 production or bacterial killing.

Upon phagocytosis, human PMN oxygen consumption increases by 100 to 300 nmol/10^8 cells per min (13, 15). Measurements of the H_2O_2 and

TABLE 3. *Total ascorbate content of human PMN; effect of time of incubation without and with latex particles*[a]

Incubation time (min)	Unstimulated	Phagocytosing
0	73	73
40	73	68
60	71	65
90	74	66
120	72	66

[a] Expressed as nmol of ascorbate/10^8 PMN.

activated oxygen species produced during phagocytosis account for up to 80% of the increment in oxygen consumption (4, 6, 12, 13, 15, 28). Despite exposure to these potent and largely diffusible denaturants, PMN survive and function at least briefly (23) and do not accumulate lipid peroxides (17) after phagocytosis. Catalase, myeloperoxidase, and glutathione peroxidase catalyze destruction of H_2O_2; glutathione peroxidase also catalyzes lipid peroxide reduction (16). Ascorbate and other small molecules, including reduced glutathione and α-tocopherol, are effective antioxidants and free radical scavengers (8, 9, 26). Human leukocytes (10^8) contain 107 to 205 nmol of reduced glutathione (11), 3.2 ± 0.2 nmol of oxidized nicotinamide adenine dinucleotide (NAD^+), 2.5 ± 0.2 nmol of NADH, 0.8 ± 0.2 nmol of $NADP^+$, and 2.4 ± 0.4 nmol of NADPH (24). The cell contents of these redox-active molecules are comparable to the ascorbate contents measured in the present study (Table 4). Human PMN can reduce more than 200 nmol of dehydroascorbate/10^8 cells per min (5). This activity would appear sufficient to maintain ascorbate in reduced form, able to act as a significant part of the PMN's capacity for inactivating free radicals and oxidants and thus for preventing denaturation of cell constituents.

Chronic granulomatous disease neutrophils phagocytose normally (25), but their ability to produce H_2O_2 and activated oxygen species is markedly impaired (4, 6). In the studies reported here, ascorbate levels were stable during phagocytosis in chronic granulomatous disease phagocytes but fell significantly in normal phagocytes. The latter observation probably reflects degradation of ascorbate, mediated by oxidants produced during phagocytosis, to compounds other than those assayable as total ascorbate. The degraded ascorbate may be sequestered from the cell's dehydroascorbate reducing activity, perhaps in phagolysosomes.

The ratio of $NADP^+$ to NADPH in PMN was observed to increase from 0.11 to 0.31 during phagocytosis (27). This has been interpreted to

TABLE 4. *Ascorbate contents of human PMN after 60-min incubation without and with latex particles*[a]

PMN	Ascorbate content (nmol/10^8 cells)		
	Total	Reduced	Dehydroascorbate
Normal[a]			
Unstimulated	78.1 ± 8.3	54.9 ± 10.3	23.2 ± 8.9
Phagocytosing	68.6 ± 4.8	40.0 ± 7.4	28.6 ± 7.6
Difference	(−)9.5 ± 4.6[b]	(−)14.9 ± 4.4[b]	(+)5.4 ± 4.6[c]
Chronic granulomatous disease[d]			
Unstimulated	54.7 52.9	46.8 44.6	7.8 8.3
Phagocytosing	54.7 53.5	41.2 43.4	13.4 10.2
Difference	0.0 (+)0.6	(−)5.6 (−)1.2	(+)5.6 (+)1.9

[a] $n = 10$; mean ± standard deviation.
[b] $P < 0.001$ by paired t test.
[c] $P < 0.005$ by paired t test.
[d] $n = 2$; recorded separately.

reflect NADPH oxidation in the process of H_2O_2 production (27) or the oxidation of reduced glutathione by H_2O_2 (4). Neutrophil-unsaturated membrane lipid, an easily oxidized cell component, is not detectably oxidized after phagocytosis (17). This suggests that extensive oxidation of cell constituents is not a general phenomenon in phagocytosing PMN. The present study demonstrates that ascorbate is oxidized during phagocytosis. This observation is consistent with the view that ascorbate is a functional part of the cell's redox-active components.

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