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Stimulation of the Hexose Monophosphate Shunt in Human Neutrophils by Ascorbic Acid: Mechanism of Action

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The addition of either ascorbic acid or dehydroascorbic acid to a suspension of polymorphonuclear leukocytes caused a dramatic increase in the resting hexose monophosphate shunt activity. A sequence of reactions involving dehydroascorbate, reduced glutathione, and reduced nicotinamide adenine dinucleotide phosphate is described to explain this stimulation. This sequence could provide an alternate method of producing H_2O_2 and a bactericidal mechanism which is independent of myeloperoxidase.

There are three well-defined metabolic phenomena which normally accompany the ingestion of a bacterium by a neutrophil, namely, increased oxygen consumption, increased production of hydrogen peroxide, and increased hexose monophosphate shunt (HMS) activity (13). Although the generation of hydrogen peroxide is generally considered crucial to the bactericidal capability of the cell, its method of formation within the cell is not at all clear. Various workers have implicated reduced nicotinamide adenine dinucleotide (NADH) oxidase (1), reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (12), and amino acid oxidases (2) as potential sources of the bactericidal H_2O_2.

The importance of hydrogen peroxide in the bactericidal activity of the neutrophil is deduced from data obtained from cells of patients with chronic granulomatous disease of childhood. In this particular syndrome, the normal increments in O_2 consumption, H_2O_2 production, and HMS activity upon phagocytosis are lacking (11). This is accompanied by defective killing of ingested bacteria.

It has been suggested that the bactericidal activity of H_2O_2 lies in its interaction with myeloperoxidase and an appropriate halide, resulting in either iodination of the microbial cell wall (14) or the production of aldehydes from the decarboxylation of amino acids (21). This requirement for a myeloperoxidase-mediated reaction for bactericidal activity is open to question, however, because patients with a complete absence of leukocyte myeloperoxidase do not suffer from recurrent bacterial infections, although moderate defects in bactericidal activity have been identified in these cells in vitro (15). Further, McCall et al. (17) reported that they completely inhibited the myeloperoxidase-mediated reactions in vitro without significantly affecting the clearance of bacteria.

We recently reported that the in vitro addition of ascorbic acid markedly stimulates the HMS in normal human neutrophils, rabbit alveolar macrophages, and in neutrophils from a patient with chronic granulomatous disease (3). The present communication describes a sequence of reactions which might explain the mechanism of the ascorbate stimulation and postulates a bactericidal mechanism which requires H_2O_2 but is independent of the reactions catalyzed by myeloperoxidase.

MATERIALS AND METHODS

Glucose-*1*-^{14}C and glucose-*6*-^{14}C (specific activities: 54.2 mCi/mmole and 36.5 mCi/mmole, respectively) were obtained from New England Nuclear Corp., Boston, Mass. The isotopes were dissolved in distilled water to give a concentration of 0.50 μCi/ml and were stored frozen. Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were used as the sodium salts. Stock solutions of ascorbic acid and dehydroascorbic acid were prepared fresh daily and neutralized to *p*H 7.0 with NaOH. Dehydroascorbic acid was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. All other biochemicals used were products of Sigma Chemical Co., St. Louis, Mo. Plasma gel, used for sedimenting red blood cells, was obtained from Laboratoire Roger Bellon, Neuilly, France.

Isolation of leukocytes. Leukocytes were isolated from the blood of apparently healthy, volunteer subjects by a method previously described (6). Samples to be used for measurement of glucose oxidation in intact cells were counted by conventional means, and the concentration was adjusted to 5×10^6 cells/ml by the addition of phosphate-buffered saline. Differential counts were done in a counting chamber by classifying cells as phagocytes (mature and band-form neutrophils, eosinophils, and monocytes) and lymphocytes. Lymphocytes accounted for less than 10% of the cell suspension. Cell viability was determined by staining with 1% trypan blue dye.

For experiments in which broken-cell preparations were used, the cells were suspended in phosphate-buffered saline and disrupted by sonic oscillation as previously described (6). The disrupted suspension was diluted to 20 ml with phosphate-buffered saline, and the protein content was estimated by the biuret method of Gornall et al. (10) with bovine serum albumin as a standard. The sonically treated material was then used in the experiments with no centrifugation procedure.

Measurement of glucose utilization. Glucose utilization in intact cells via the Krebs cycle and the HMS was estimated by a procedure previously described, with the use of glucose differentially labeled in the C-1 or C-6 position (7).

Glucose utilization by broken-cell preparations was assayed by a modification of this procedure. Each flask contained a total volume of 2.70 ml consisting of 1.6 ml of phosphate-buffered saline, 0.10 ml of isotope (0.20 μCi of either glucose-*1*-^{14}C or glucose-*6*-^{14}C), and 1.0 ml of the sonically treated leukocyte suspension containing 0.88 mg of protein. Various compounds were dissolved in the buffer and added to a final concentration of 0.22 mg/ml. The reaction was initiated by the addition of sonically treated material and allowed to proceed for 1 hr at 37 C. The procedure for collection and counting of $^{14}CO_2$ was the same as previously described (11).

Determination of GSH. The reaction between dehydroascorbic acid and reduced glutathione (GSH) was quantitated spectrophotometrically by the use of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; 5). Each incubation tube contained the following: 100 μmoles of phosphate buffer (*p*H 7.4), 3 μmoles of GSH, and deionized water to give a final volume of 1.0 ml. When present, dehydroascorbic acid was added to give a final concentration of 3 mM. Tubes were incubated at 37 C for various periods of time. The reaction was terminated by pipetting a 0.10-ml sample into 10.0 ml of cold 0.02 M phosphate buffer, *p*H 8.0. Free sulfhydryl groups were detected by the addition of 0.05 ml of DTNB (0.396 g/100 ml) followed by incubation at room temperature for 30 min. The absorbance at 410 nm was related to GSH concentration by reference to a standard curve which was linear from 0.00 to 0.50 μmole of GSH.

Bactericidal assay. *Escherichia coli* (Seattle) and *Staphylococcus aureus* (Seattle) were cultured overnight in Trypticase Soy Broth, centrifuged, washed, and diluted to a standard absorbance value (0.08 at 525 nm). When *S. aureus* was the test organism, this suspension was used undiluted; when *E. coli* was the test organism, it was necessary to use a 1:10 dilution of this stock bacterial suspension. L-Ascorbic acid was freshly prepared and neutralized to a *p*H of 7.4. The complete reaction mixture contained, in a final volume of 3.0 ml: phosphate buffer (*p*H 7.0), 100 μmoles; L-ascorbic acid, 20 μmoles; H_2O_2, 0.08 μmole; $CuSO_4$, 0.01 μmole; and bacteria, 1.70 ml of the standard suspension. The mixtures were incubated in a shaker bath at 37 C for 30 min, and the number of viable bacteria was determined by the plate dilution technique of Maaløe (16).

RESULTS

Table 1 illustrates the effect of ascorbic acid and dehydroascorbic acid on the HMS activity of intact human polymorphonuclear leukocytes.

The addition of 0.01 M ascorbate to a suspension of resting cells resulted in a marked stimulation of the HMS, in accord with previous observations (3). A similar concentration of dehydroascorbate (DHA) resulted in a significantly greater stimulation of the HMS, suggesting that this compound might be an intermediate in the stimulation by ascorbate. The stimulatory effect of 0.01 M DHA in these experiments is as large in magnitude as that observed when the cells are actively phagocytizing polystyrene particles.

In an attempt to define further the mechanism of the HMS stimulation by ascorbate, we turned to experiments employing broken-cell preparations and tested the effects of a number of

TABLE 1. *Effect of ascorbate and dehydroascorbate on hexose monophosphate shunt activity of intact polymorphonuclear leukocytes*

Description	Counts/min in $^{14}CO_2$ from glucose-*1*-^{14}C[a]	
	Expt 1	Expt 2
Resting cells	1,140 (1,139–1,192)	1,003 (915–1,101)
+ Polystyrene particles	7,436 (7,044–8,092)	11,113 (10,359–11,912)
+ Ascorbate (0.01 M)	4,203 (3,993–4,368)	4,130 (3,973–4,351)
+ Dehydroascorbate (0.01 M)	7,916 (7,804–8,036)	9,331 (9,218–9,465)

[a] All values are corrected for controls in which glucose-*6*-^{14}C was used. Each value represents the average of three determinations; numbers in parentheses represent the range.

TABLE 2. *Effect of various metabolic intermediates on hexose monophosphate shunt activity of leukocyte sonic extracts*

Description	Counts/min in $^{14}CO_2$ from glucose-1-^{14}C[a]	
Control	176	(168–185)
+ NADP	3,386	(3,308–3,495)
+ Ascorbate (ASC)	184	(172–188)
+ Dehydroascorbate (DHA)	212	(204–238)
+ Reduced glutathione (GSH)	185	(181–195)
+ Oxidized glutathione (GSSG)	189	(184–195)
+ NADPH	345	(317–369)
+ NADPH + GSH	895	(870–917)
+ NADPH + GSSG	3,984	(3,793–4,194)
+ DHA + GSH	208	(196–228)
+ DHA + NADPH	428	(419–440)
+ DHA + GSH + NADPH	4,808	(4,694–4,844)
+ ASC + GSH + NADPH	1,075	(993–1,125)

[a] All values are corrected for controls in which glucose-6-^{14}C was used. Each value represents the average of three determinations; numbers in parentheses represent the range.

metabolic intermediates on the HMS activity in this system (Table 2). A large stimulation of the HMS (20 times) was observed when the system was fortified with NADP, indicating that availability of this cofactor is rate-limiting. Neither ascorbate nor DHA alone stimulated the HMS, indicating that these substances cannot simply substitute for NADP, i.e., act as carriers of H^+ and electrons. A strong stimulation of the HMS was observed when both NADPH and oxidized glutathione (GSSG) were added simultaneously, owing to the presence of an NADPH-dependent glutathione reductase in the white cell (20). The intermediate stimulation observed with NADPH and GSH is probably due to air oxidation of some of the GSH to GSSG followed by the glutathione reductase reaction. Similarly, a strong stimulation of the HMS was obtained with a combination of DHA, GSH, and NADPH. The intermediate stimulation observed here when ascorbate is substituted for the DHA might be due to autoxidation of some of the ascorbate to the latter compound. All other combinations of cofactors tested gave no significant stimulation of HMS activity.

The stimulation observed with the combination of DHA, GSH, and NADPH suggested the possibility that the DHA was first reacting with GSH to give GSSG plus ascorbate. The GSSG

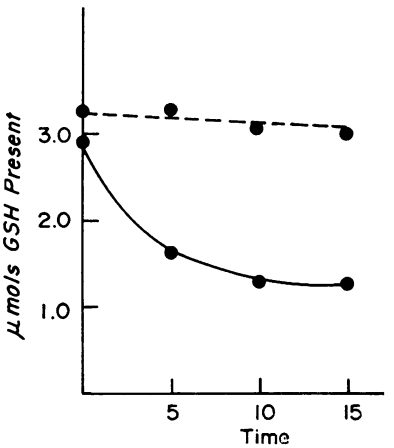

FIG. 1. *Reaction of reduced glutathione (GSH) with dehydroascorbate. Dashed line: control, no dehydroascorbate. Solid line: 3 mM dehydroascorbate added. Samples of the reaction mixture were removed at the time intervals shown and assayed for GSH as described in Materials and Methods.*

thus generated could then react with NADPH in the glutathione reductase reaction.

To test this hypothesis, GSH was incubated in the presence and absence of DHA, and the amount of GSH present was determined at several time intervals as described in Materials and Methods. The results in Fig. 1 indicate that a very small amount of GSH was oxidized during a 15-min incubation in the absence of DHA, but a very rapid reaction occurred upon the addition of the DHA. Under these conditions, almost half of the glutathione present was oxidized in the first 5 min. The reaction was shown to be spontaneous; the addition of a sonic extract of leukocytes caused no increase in the rate of the reaction, indicating that the reaction was nonenzymatic (data not shown).

To assess the potential physiological significance of the reactions, the effect of ascorbic acid on the bactericidal capability of the neutrophil was tested. Table 3 demonstrates the in vitro bactericidal activity of 6.7 mM ascorbic acid against *E. coli* and *S. aureus*. Ascorbic acid alone had a significant bactericidal activity after incubation at 37 C for 30 min. *E. coli* was destroyed somewhat more readily than *S. aureus* under all conditions tested. The bactericidal activity of ascorbate was enhanced considerably by the addition of both H_2O_2 and Cu^{++}. No bactericidal activity was demonstrated by these concentrations of H_2O_2 or Cu^{++}, either alone or in combination. A similar experiment in which a lower concentration of ascorbate (0.67 mM) was

TABLE 3. *Bactericidal activity of ascorbic acid*

Conditions	No. of viable colonies[a]	
	E. coli	*S. aureus*
No additions	3,300,000 (2,800,000–3,800,000)	2,100,000 (1,200,000–3,600,000)
Ascorbic acid (6.7 mM)	43,000 (20,000–86,000)	130,000 (60,000–170,000)
Ascorbic acid (6.7 mM) + H_2O_2 (0.027 mM) + Cu^{++} (0.003 mM)	10(0–10)	9,000 (1,000–49,000)

[a] Values represent the mean of duplicate determinations in three separate experiments. Numbers in parentheses represent the range.

used gave the same qualitative results (data not shown).

DISCUSSION

The data suggest a sequence of reactions which might explain the ascorbate-induced increases in O_2 consumption and HMS activity.

I. ascorbate + O_2 → DHA + H_2O_2
II. ascorbate + H_2O_2 + $2H^+$ → DHA + $2H_2O$
III. DHA + 2GSH → ascorbate + GSSG
IV. GSSG + NADPH + H^+ → 2GSH + NADP
V. glucose-6-phosphate + NADP → HMS stimulation

The first reaction (8) might well be involved in the physiology of the leukocyte. The reaction is nonenzymatic in nature and is catalyzed by small amounts of heavy metal ions, e.g., Cu^{++}. Such a reaction could serve as an alternate source of H_2O_2 for the cell and might be responsible, at least in part, for the increases in O_2 consumption and H_2O_2 production which normally accompany the phagocytic process.

In reaction II, a second molecule of ascorbate reacts with the H_2O_2 generated in reaction I (or H_2O_2 generated from other sources, e.g., NADH oxidase, amino acid oxidase, etc.) to yield DHA and water. This reaction can be observed directly by following the absorbance due to ascorbate at 260 nm. It is a rapid, nonenzymatic reaction which proceeds faster at *p*H 5 than at *p*H 7 (*unpublished data*).

Antimicrobial activity may be apparent during both reactions I and II. Bacteria, viruses, and fungi are killed by the oxidation of ascorbic acid (9). Miller recently demonstrated that a reaction between ascorbic acid and H_2O_2 gives rise to an intermediate which is bactericidal in vitro (18). The results presented in Table 3 confirm these observations. This potential mechanism of bacterial killing is independent of myeloperoxidase and might explain the normal bacterial clearance observed under conditions in which myeloperoxidase is completely inhibited.

The remaining reactions (III–V) serve to explain the HMS stimulation by ascorbate and have been inferred from the data presented here. The HMS stimulation in this sequence follows the generation of H_2O_2 and is dependent upon it. This is consistent with the work of Reed (19), who demonstrated the stimulatory effect of H_2O_2 on the HMS in neutrophils. Since DHA is an intermediate in this sequence, the observation that DHA causes a greater stimulation of the HMS than does ascorbate itself is expected.

The experiments in which broken-cell preparations were used lend support to this sequence of reactions. Under these conditions, maximal stimulation of the HMS is observed when DHA, GSH, and NADPH are added in concert to a sonic extract of neutrophils. This stimulation is not observed when any two of the three are added, indicating that all three compounds must take part in the stimulation. GSSG can, however, substitute for the combination of GSH and NADPH, suggesting that the glutathione reductase (20) reaction is one of the steps in the sequence (reaction IV). The other postulated reaction in the sequence (reaction III) was demonstrated by measuring the disappearance of GSH in the presence of DHA.

This sequence of reactions, then, can adequately explain the increases in O_2 consumption and HMS activity which occur when ascorbate is added to resting cells. It can also supply an alternate source of H_2O_2 to the cell and a mechanism of bacterial killing which is independent of myeloperoxidase.

Whether these reactions are involved in the respiratory burst which normally accompanies phagocytosis is a matter for further study. The neutrophil actively accumulates ascorbic acid to a final concentration of 1 to 3 mM (4), suggesting that this compound might be important in the normal function of the neutrophil. One difficulty with postulating a physiological role for these reactions lies in the initiation of the sequence; this might be explained by a change in the compartmentalization of the reactants after phagocytosis.

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