Ascorbate-mediated stimulation of neutrophil motility and lymphocyte transformation by inhibition of the peroxidase/H_2O_2/halide system in vitro and in vivo[1]

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ABSTRACT Neutrophil migration, postphagocytic hexose-monophosphate shunt activity and myeloperoxidase-mediated iodination of ingested *Candida albicans* and lymphocyte mitogen-induced transformation were assessed in six normal volunteers before and 1 h after a single intravenous injection of 1 g ascorbate. Increased neutrophil motility was observed which was associated with decreased myeloperoxidase-mediated iodination of *C. albicans* and a slight increase in hexose-monophosphate shunt activity. Lymphocyte transformation was also increased. Alterations in these activities were related to serum ascorbate levels. To investigate the relationship of ascorbate-mediated increased neutrophil motility and lymphocyte transformation to decreased peroxidase activity neutrophils and lymphocytes from normal individuals were exposed to the horseradish peroxidase/H_2O_2/sodium iodide system in the presence and absence of ascorbate and tested for migratory and proliferative responses respectively. Exposure to the horseradish peroxidase/H_2O_2/halide system caused inhibition of neutrophil motility and lymphocyte responsiveness to mitogens. However, inclusion of ascorbate protected both the neutrophils and lymphocytes from the inhibitory effects of the horseradish peroxidase/H_2O_2/halide system. *Am. J. Clin. Nutr.* 34; 1906–1911, 1981.

KEY WORDS Ascorbate, myeloperoxidase, hydrogen peroxide, lymphocyte transformation, neutrophil motility, immunostimulation

Introduction

Ascorbate has been reported to increase motility of human neutrophils in vitro (1) and in vivo (2) and lymphocyte transformation in mice in vivo (3). We have recently found that ingestion of 2 to 3 g ascorbate daily in normal adults caused enhanced neutrophil migration; ingestion of 1 to 3 g ascorbate daily was associated with increased lymphocyte responsiveness to mitogens (4). These increased activities were accompanied by decreased activity of the myeloperoxidase (MPO)/H_2O_2/halide system. To investigate possible relationships between ascorbate-mediated inhibition of the MPO/H_2O_2/halide system and increased neutrophil motility and lymphocyte transformation the previous studies were repeated before and 1 h after intravenous injection of 1 g ascorbate. This procedure was used to rapidly achieve high serum levels of ascorbate.

In a further series of experiments neutrophils and lymphocytes were exposed to the horseradish peroxidase (HRP)/H_2O_2/halide system in the presence and absence of sodium ascorbate over a concentration range of 10^{-5} to 10^{-1} *M*. After washing the processed neutrophils and lymphocytes were tested for migratory and proliferative activities, respectively.

Methods

Testing of neutrophil and lymphocyte functions was performed on six healthy individuals before and 1 h after a single intravenous injection of 1 g ascorbate in a total injectable volume of 5 ml (Redoxon, Hoffman—La Roche and Co. Ltd., Basle, Switzerland).

Neutrophil functions

Studies of motility. Polymorphonuclear neutrophilic leukocytes (PMN) were obtained from heparinized ve-

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nous blood (5 U heparin/ml) and were resuspended to a final concentration of 3×10^6/ml in HEPES (N-2-hydroxy-ethylpiperazine-N-2-ethanesulfonic acid; Sigma Chemical Co. St. Louis, MO)-buffered Hanks' balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, NY) supplemented with 0.05% bovine serum albumin after hypotonic lysis of residual erythrocytes with 0.84% ammonium chloride.

One leukoattractant was used viz. fresh autologous serum activated with 100 μg of bacterial endotoxin (*Escherichia coli* 0127:B8; Difco Laboratories, Detroit, MI) per milliliter which was diluted 8-fold with HBSS before use. The assays of motility were performed in modified Boyden chambers (5) with 5 μm pore size membrane filters (Sartorius-membranfilter, Göttingen, West Germany) and a 2-h incubation period. The results are expressed as the number of cells per microscope high power field as an average of triplicate filters.

Phagocytic studies. In studies of postphagocytic metabolic activity the cell suspending medium used was 0.15 *M* phosphate-buffered saline pH 7.2 These activities were stimulated using *Candida albicans* at concentrations which in our hands give maximal stimulation of postphagocytic metabolic activity. Pure PMN suspensions prepared as previously described (4) of > 90% purity and viability were used in these studies.

Hexose monophosphate shunt activity was measured according to the method of Wood et al. (6) with minor modifications (4) by potassium hydroxide absorption of $^{14}CO_2$ derived from glucose radiolabeled in the 1-C position, (D-glucose 1– ^{14}C, New England Nuclear, Boston, MA). The reaction mixture contained 2×10^6 PMN (0.1 ml), 0.1 ml autologous serum, 1×10^7 *C. albicans* (0.1 ml), 0.1 ml of phosphate-buffered saline and 0.6 ml radiolabeled glucose containing 0.06 μCi. Incubation was for 1 h after which the reaction was terminated and CO_2 released by the addition of 2 ml of 1 N HCl. After another hour the radioactivity associated with KOH was determined in a liquid scintillation counter. Results are expressed as nmoles glucose metabolized/60 min.

MPO-mediated iodination of ingested protein was determined by the method of Root and Stossel (7) with minor modifications. To 0.1 ml of PMN suspension (1×10^7/ml) was added 0.1 ml of *C. albicans* (1×10^8/ml), 0.1 ml of fresh autologous serum, 0.1 ml of an I^{125} solution (0.6 μCi/ml) (New England Nuclear, sodium iodate), and 0.6 ml of phosphate buffered saline. Incubation was for 60 min/37°C on a turntable after which the extent of incorporation of I^{125} into acid precipitable protein was determined by solid scintillation counting. Results are expressed as nmoles I^{125} in the protein precipitate. To assess if the observed effects on MPO-mediated iodination were cell or serum-mediated, different combinations of pre- and postascorbate neutrophils and serum were investigated.

Lymphocyte transformation. Blood for these studies was defibrinated and fractionated by density gradient centrifugation (Ficoll:sodium metrizoate gradients) at 400 g/25 min. The mononuclear cell layer was removed and twice washed in TC 199 (Grand Island) pH 7.2 supplemented with HEPES (Sigma) 2 g/l and heat inactivated autologous serum. The cell suspension was adjusted to 4×10^6 mononuclear cells/ml. Aliquots of 50 μl (2×10^5 cells) were placed in wells of 5 mm Linbro tissue culture plates (Flow Laboratories, Inglewood, CA) together with 100 μl of serum supplemented TC 199. The mitogens used in this study were phytohemagglutinin (PHA), Wellcome Reagents, Ltd., Beckenham, England) and concanavalin A (Sigma) at concentrations of 25 and 50 μg/ml. Mitogens were added in 20 μl volumes to triplicate wells and unstimulated controls received 20 μl of TC 199. The final volume in each well was brought to 200 μl by the addition of serum supplemented TC 199. The plates were mixed and incubated for 48 h in a humidified atmosphere of 3% CO_2 in air after which 20 μl of tritiated thymidine (^{3}H-T. thymidine-methyl-^{3}H. New England Nuclear) containing 0.2 μCi was added to each well and the plates incubated for a further 18 h. Harvesting was performed using a multiple automated sample harvester (MASH-II, Microbiological Associates, Bethesda, MD). Incorporation of ^{3}H-T was assessed in a liquid scintillation spectrophotometer. To investigate possible serum effects the mitogen-induced reactivity was measured in the presence of 25 and 50% (final concentration) preascorbate and postascorbate serum.

Effects of the HRP/H_2O_2/halide system on neutrophil migration. In these studies 3×10^6 neutrophils were incubated with 0.27 units of HRP (HRP, Grade I, Sigma Chemical Co.), 1 m*M* sodium iodide, 1 μ*M* H_2O_2 in the presence and absence of sodium ascorbate over a concentration range of 10^{-5} to 10^{-1} *M*. The final reaction volume was 1 ml HBSS. After 15 min incubation the reaction mixture was diluted with five parts HBSS supplemented with 0.1% bovine serum albumin and the cells washed and resuspended to 3×10^6/ml in bovine serum albumin supplemented HBSS. The cells were then tested for chemotactic responsiveness to autologous EAS.

Effects of the HRP/H_2O_2/halide system on lymphocyte transformation. In these studies 4×10^6 mononuclear cells in TC 199 were exposed to the HRP/H_2O_2/halide system in the presence and absence of sodium ascorbate at concentrations of 10^{-5} to 10^{-1} *M*. After 15 min incubation the reaction mixture was diluted with five parts of TC 199 supplemented with autologous serum and the cells washed and resuspended to 4×10^6/ml and tested for responsiveness to both mitogens. In control systems lymphocytes were exposed to HRP only or H_2O_2 and sodium iodide only.

Results

Results are expressed as the mean value of six separate experiments with the SE. Statistical analysis was performed by the Student's *t* test for 2 means.

Neutrophil functions

Significantly increased neutrophil motility to autologous EAS was observed after injection of ascorbate which was related to significantly decreased MPO-mediated iodination (**Table 1**). No significant effects on hexose monophosphate shunt activity were found. To assess if effects on MPO activity were cell or serum mediated, combinations of pre- and postascorbate neutrophils and serum

were investigated (**Table 2**). These results show that the effects on inhibition of MPO-mediated iodination are caused solely by serum ascorbate since postascorbate neutrophils with preascorbate serum caused no inhibition.

The effects on lymphocyte transformation to 25 μg/ml PHA of high dose ascorbate are shown in **Table 3.** Stimulation of transformation was achieved when 25% postascorbate serum was used. However 50% postascorbate serum caused inhibition of the proliferative response to PHA. Similar results were achieved with Con A (results not shown).

The HRP/H_2O_2/halide system mediated complete inhibition of neutrophil motility to EAS and partial inhibition of lymphocyte responsiveness to both mitogens which was 35% ± 8.1%, 41% ± 11.2%, 44% ± 8.3%, and 42% ± 9.8% for 25 and 50 μg PHA and Con A, respectively. However, sodium ascorbate protected both the neutrophil migratory responsiveness (**Fig. 1**) and the lymphocyte blastogenic response to PHA and Con A (**Fig. 2**) from inactivation by the HRP/H_2O_2/halide system. No inhibition of mitogen-induced lymphocyte transformation was observed in control systems containing HRP only or H_2O_2 and sodium iodide only.

Discussion

This study has demonstrated a relationship between ascorbate-mediated inhibition of the MPO/H_2O_2/halide system and stimulation of neutrophil motility in vivo. In vitro experi-

TABLE 1
Neutrophil migration and postphagocytic hexose monophosphate shunt (HMS) activity and MPO-mediated protein iodination before and after intravenous sodium ascorbate

	Serum ascorbate	Neutrophil migration to autologous endotoxin activated serum (results as cells/HPF)	HMS activity as nmoles C^{14}-glucose metabolized	MPO-mediated iodination as nmol I^{125}
	μg/ml			
Before ascorbate	11.9 ± 1.3*	66 ± 17	17 ± 3.1	0.92 ± 0.05
After	44.8 ± 4.2	127 ± 33	19.4 ± 4.2	0.68 ± 0.05
		($p < 0.02$)	NS	($p < 0.05$)

* Results as mean response with SE in six adult controls.

TABLE 2
Assessment of pre and postascorbate serum and neutrophil-mediated inhibition of MPO iodination of ingested *C. albicans*

Source of neutrophils	Source of serum	MPO-mediated iodination of *C. albicans* as nmol I^{125} precipitated	% Inhibition of iodination
Preascorbate	Preascorbate	0.81 ± 0.06*	
Preascorbate	Postascorbate	0.65 ± 0.03†	20
Postascorbate	Preascorbate	0.80 ± 0.05	1
Postascorbate	Postascorbate	0.61 ± 0.04†	24

* Results as mean and standard error of six separate experiments.
† p value < 0.05.

TABLE 3
Lymphocyte transformation to 25 μg/ml PHA in six normal volunteers before and 1 h after intravenous ascorbate

Source of lymphocytes	Source and concentration of serum in the culture system	Responsiveness of the lymphocytes to 25 μg/ml PHA
Preascorbate (10.6 μg/ml)†	25% Preascorbate*	23.954 ± 7.23‡
Postascorbate (44.8 μg/ml)†	25% Postascorbate	33.217 ± 4.703§
Preascorbate	50% Preascorbate	37.300 ± 3.842
Postascorbate	50% Postascorbate	28.441 ± 6.612

* Autologous serum was used for these studies.
† Mean serum ascorbate values.
‡ Results as mean cpm and SE of six experiments.
§ p value < 0.05.

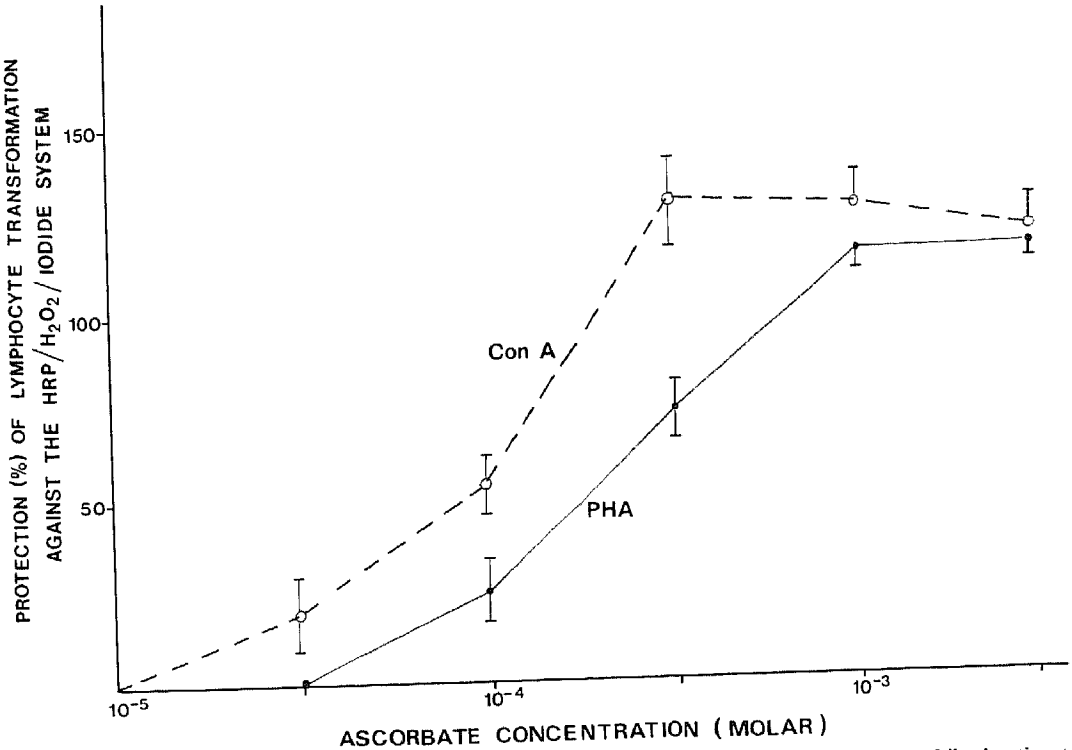

FIG. 1. Effects of ascorbate on the HRP/H_2O_2/halide system mediated inhibition of neutrophil migration to EAS in vitro.

ments using the HRP/H_2O_2/sodium iodide system confirmed the relationship. Preincubation of neutrophils with the HRP/H_2O_2/halide system eliminated the migratory responsiveness of treated neutrophils to autologous EAS. However inclusion of ascorbate (at concentrations of $>10^{-3}$ *M*) caused a dose-dependent reduction in the degree of inhibition of neutrophil migration mediated by the HRP/H_2O_2/halide system. The extent of in vivo inhibition of MPO-mediated iodination was related entirely to the serum ascorbate concentration. This is in agreement with our previous findings that ascorbate-mediated stimulation of neutrophil migration is a serum-dependent phenomenon (8) and that blood neutrophils of normal individuals do not concentrate ascorbate (9). We have recently observed that neutrophils incubated with the leukoattractants EAS and N-formyl-L-methionyl-L-methionyl-L-phenylalanine in the presence of radiolabeled ascorbate do not actively take up the ascorbate (R. Anderson, unpublished observations). The most likely mechanism of ascorbate-mediated stimulation of neutrophil motility is inhibition of autoxidation of the neutrophil membrane by MPO and H_2O_2 released from neutrophils after exposure to leukoattractants (10, 11). Ascorbate in the extracellular milieu could protect the neutrophil membrane from attack by the MPO/H_2O_2/halide system. It is important that in vitro determinations of immunological functions following ascorbate ingestion should use autologous serum.

Increased lymphocyte transformation was observed with 25% postascorbate serum in the culture system. However inclusion of 50% postascorbate serum was associated with decreased lymphocyte responsiveness to mitogens. This indicates that high serum levels of ascorbate (>44 μg/ml) can cause decreased lymphocyte proliferation. However, further in vivo experimentation is required to assess the significance of this observation in relation to megadose ascorbic acid usage. As with neutrophils lymphocyte transformation to mitogens was inhibited in vitro by exposure of lymphocytes to the HRP/H_2O_2/halide system. Inclusion of ascorbate in the reaction

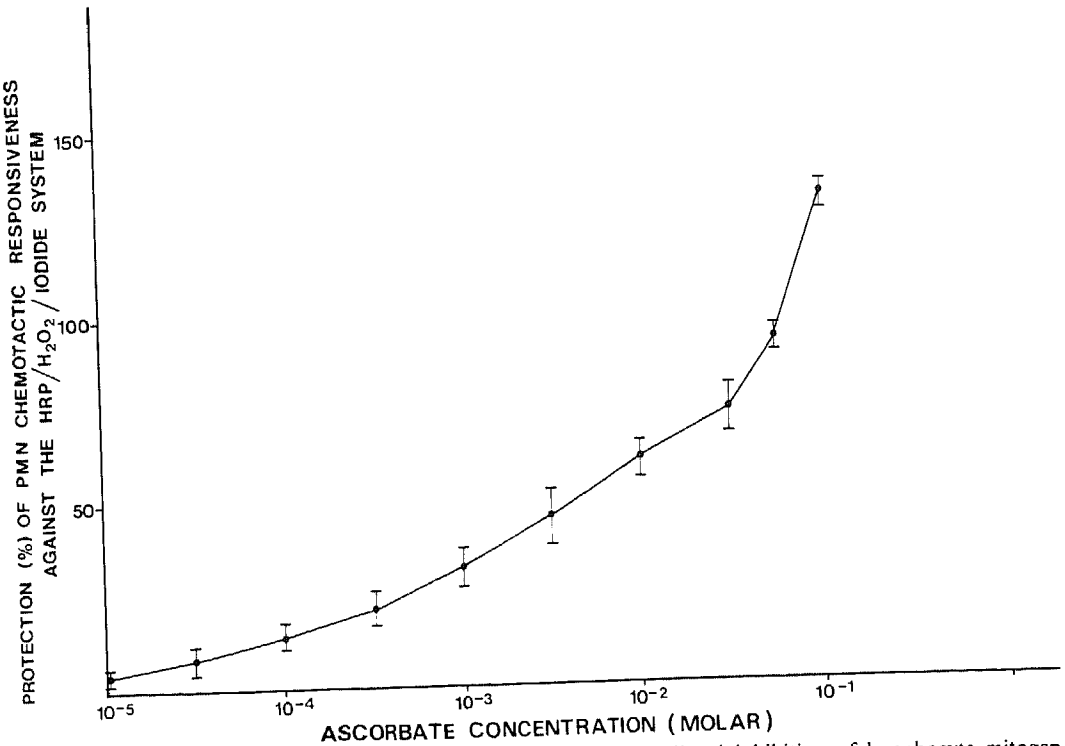

FIG. 2. Effects of ascorbate on the HRP/H_2O_2/halide system mediated inhibition of lymphocyte mitogen-induced transformation in vitro.

system protected the lymphocyte proliferative response from inactivation by the HRP/H_2O_2/halide system. Coculturing of lymphocytes with neutrophils or neutrophil products released by sonication of neutrophils causes inhibition of lymphocyte transformation (12). It is possible that MPO and H_2O_2 release by neutrophils inhibits lymphocyte transformation in vivo and in vitro. The degree of this inhibition could be reduced by inhibitors of the peroxidase/H_2O_2/halide system such as ascorbate (13, 14). The degree of inhibition of lymphocyte transformation caused by the peroxidase/H_2O_2/halide system was less than that observed for neutrophil migration. This may reflect a differential sensitivity of neutrophils and lymphocytes to oxidative damage. Alternatively lymphocytes may partially recover from peroxidase mediated damage during the 70-h incubation period.

The most probable mechanism of ascorbate-induced stimulation of neutrophil motility in vitro and in vivo and lymphocyte transformation in vivo is protection of these functions from inhibition by the peroxidase/H_2O_2/halide system. Ascorbate may inhibit activity of peroxidase enzymes. A second possibility is that oxidisable groups on the ascorbate molecule may compete with similar groups on neutrophil and lymphocyte membranes for toxic oxidative intermediates generated by the peroxidase/H_2O_2/halide system. This system is activated during neutrophil migration (10) and phagocytosis (15).

There is disagreement as to the benefits and risks of megadose ascorbic acid usage. However, the findings of this study suggest a mechanism by which ascorbate could be useful immunotherapeutically and immunoprophylactically in immunocomprised individuals, especially those with reduced neutrophil migratory and/or lymphocyte proliferative responses. Ascorbate by preventing autooxidation of leukocyte membranes could protect and potentiate neutrophil and lymphocyte functions, although possible inhibitory effects of high-dose ascorbate therapy on lymphocyte proliferation should be considered.

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