

# The effects of increasing weekly doses of ascorbate on certain cellular and humoral immune functions in normal volunteers<sup>1</sup>

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**ABSTRACT** Certain functions of the blood neutrophils and lymphocytes from normal adult volunteers were evaluated after the ingestion of increasing doses of ascorbate. Serum immunoglobulins and levels of C'3 and C'4 and total hemolytic complement were also measured. Enhancement of neutrophil motility to a chemotactic stimulus of endotoxin-activated autologous serum was observed in normal adult volunteers after the ingestion of 2 and 3 g ascorbate daily. No alteration was observed at lower doses. Other neutrophil functions evaluated that remained unaltered by ascorbate, were postphagocytic hexose monophosphate shunt activity and myeloperoxidase mediated iodination of ingested protein. Stimulation of lymphocyte transformation to the mitogens phytohaemagglutinin and concanavalin A was detected after the daily ingestion of 1, 2, and 3 g of ascorbate. Mitogen-induced protein synthesis was unaffected. Serum levels of IgG, IgA, IgM, C'3, and C'4 and total complement activity were unaltered by ascorbate. *Am. J. Clin. Nutr.* 33: 71-76, 1980.

Certain cellular immune functions in vivo and in vitro and humoral factors can be altered by vitamin C. The motility of normal neutrophils (1) and monocytes (2) is enhanced by ascorbate. Correction of abnormal neutrophil motility after ingestion of ascorbate has been reported (3). Ascorbic acid mediates stimulation of polymorphonuclear leukocyte (PMN) hexose monophosphate shunt activity (HMS) (4) and antimicrobial activity in cell free systems in vitro (5) but inhibits myeloperoxidase-mediated iodination of ingested protein (6). The in vivo effects of ascorbate on postphagocytic metabolic activity are controversial; enhancement of animal (7) HMS activity by ascorbate has been reported. However Shiloh and Bhat (8) have reported that high doses of vitamin C caused a depression of the bactericidal capacity of normal human PMN.

Lymphocyte functions are also reportedly affected by vitamin C. In vitro inhibition of human blood lymphocyte transformation to phytohemagglutinin (PHA) by ascorbate at fairly high concentrations of ascorbate has been described (9). However, substantial stimulation of lymphocyte transformation after the ingestion of vitamin C in mice has been reported (10). Ascorbate can also me-

diate enhancement of interferon production in vivo (11) and in vitro (12).

Prinz et al. (13) have reported that the levels of IgA and IgM and C'3 but not of IgG and C'4 were raised in the serum of normal individuals who ingested ascorbate daily over a prolonged period.

The present study was undertaken to assess the simultaneous effects of increasing doses of ascorbate on certain cellular and humoral immune parameters. The cellular functions assessed were the in vitro kinetics of neutrophil chemotactic responsiveness and postphagocytic hexose monophosphate shunt activity and myeloperoxidase-mediated protein iodination; lymphocyte transformation to the mitogens PHA and Con A and PHA induced protein synthesis. Serum immunoglobulins IgG, IgA, and IgM and C'3, C'4, and total hemolytic complement were also assessed.

## Methods

Cellular and humoral immune testing was performed on five healthy adults before the ingestion of vitamin C. These individuals then ingested 1 g of ascorbate daily for 1 week, 2 g daily for the 2nd week, and 3 g daily for

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the 3rd week. Repeat in vitro testing of immune function was performed after each week before increasing the dose of vitamin C and 1 week after the cessation of ascorbate ingestion. Ascorbate was kindly supplied by Roche S.A. (Pty) Limited as soluble 1 g tablets.

#### Neutrophil functions

**Chemotaxis.** Blood for neutrophil chemotactic studies was taken into heparin (5 units/ml), centrifuged briefly at  $300 \times g$  for 10 min and the plasma and upper red cell and leukocyte-rich layers removed and sedimented at 37 C for 30 min after which the buffy layer was removed and lightly centrifuged ( $250 \times g$  for 10 min). The supernatant was removed and the cell pellet washed twice with Hank's balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, N.Y.) and cells resuspended to  $3 \times 10^6$  neutrophils per milliliter. Only one leukoattractant was used in this study, viz. autologous serum activated with 500  $\mu\text{g/ml}$  bacterial endotoxin (*Escherichia coli* O127:B8, Difco, Detroit, Mich.). Chemotactic responsiveness was evaluated using the method of Boyden (14) with modified chemotactic chambers (15) that were incubated at 37 C for 3 hr after which filters were removed, processed, inverted, and the number of cells that achieved complete trans-filter passage enumerated microscopically. Using this technique results were expressed as cells per microscope high power field.

#### Studies of postphagocytic metabolic activity

**Hexose monophosphate shunt activity.** The extent of HMS activity was assessed according to the method of Wood et al. (16) with minor modifications. Neutrophils from heparinized venous blood were separated from mononuclear cells by density gradient centrifugation (Ficoll: sodium metrizoate gradients) at  $400 \times g/15$  min. The resultant erythrocyte/neutrophil fraction was sedimented with 3% gelatin for 30 min at 37 C to remove most of the red cells.

The neutrophil-rich supernatant was centrifuged at  $250 \times g$  for 10 min and the erythrocytes in the cell pellet lysed with hypotonic (0.85%) ammonium chloride. The neutrophil-rich supernatant was centrifuged and washed once with 0.15 M phosphate buffered saline (PBS) and resuspended to  $1 \times 10^7$  PMN per milliliter. The assay system employed 0.2 ml PMN suspension ( $2 \times 10^6$  PMN), 0.1 ml autologous serum, 0.1 ml of a *Candida albicans* suspension containing  $1 \times 10^6$  organisms per milliliter, (cell:*C. albicans* ratio 1:5) and 0.6 (0.06  $\mu\text{Ci}$ ) ml of glucose radiolabeled in the C<sup>1</sup> position (New England Nuclear, Boston, Mass., as D-glucose 1-<sup>14</sup>C). In control systems 0.1 ml of PBS substituted for *C. albicans* and the isotope control contained 0.4 ml of PBS and 0.6 ml of radiolabeled glucose. The apparatus used to test HMS activity is a double chambered stoppered system in which the outer chamber contains the neutrophils and *C. albicans* and the inner chamber contains 0.6 ml of 1 N KOH. Each chamber was incubated at 37 C for 1 hr after which the reaction was terminated by the addition of 2 ml of 1 N HCl to the outer chamber. The chambers were allowed to stand for a further hour at room temperature to permit absorption of released <sup>14</sup>CO<sub>2</sub> by the KOH. After this time 0.2 ml of KOH was removed and added to 3 ml of scintillation fluid (Insta-gel acidified with 5.5 ml of 17 N HCl per liter). HMS activity was

expressed as corrected radioactive cpm after deduction of the isotope control and unstimulated controls.

**Protein iodination.** Determination of postphagocytic iodination of ingested *C. albicans* was performed according to the method of Root and Stossel (17). Pure neutrophil suspensions were used and adjusted to a final count of  $1 \times 10^7$  PMN per milliliter. Each experimental tube contained 0.1 ml of cell suspension ( $1 \times 10^6$  PMN), 0.1 ml of *C. albicans* ( $1 \times 10^7$  organisms, to give a PMN:*C. albicans* ratio of 1:10), 0.1 ml of autologous serum, 0.1 ml of <sup>125</sup>I (New England Nuclear, sodium iodate) solution in PBS (0.6  $\mu\text{Ci/ml}$ ) and 0.6 ml of PBS. Controls were of two types: 1) cell and *C. albicans* control in which the serum was omitted and, 2) corresponding serum control in which the cells and *C. albicans* were omitted.

Tubes were rotated on a turntable for 1 hr at 37 C after which protein was precipitated by the addition of 2 ml of 10% perchloric acid. The protein precipitate was centrifuged down and twice washed with 2 ml of 10% perchloric acid. The amount of protein associated <sup>125</sup>I was determined by solid scintillation counting. Results were expressed as corrected cpm for experimental tubes by summing the average counts from the two controls and deducting this value from the corresponding experimental value.

For both HMS and protein, iodination determination assay systems were always run in duplicate for each time interval tested. As for cell motility studies, corresponding neutrophils and autologous sera were matched for each time interval.

#### Lymphocyte studies

**Mitogen-induced DNA synthesis.** Blood for studies of lymphocyte function was defibrinated and fractionated by density gradient centrifugation (Ficoll: sodium metrizoate gradients) at  $400 \times g$  for 25 min. The mononuclear cell layer was removed and twice washed in medium RPMI 1640 pH 7.2 (Grand Island Biological Co.) supplemented with HEPES (Sigma, St. Louis, Mo.) 2 g/liter and 10% heat inactivated autologous serum. The cell suspension was adjusted to  $2 \times 10^6$  mononuclear cells per milliliter. Aliquots of 50  $\mu\text{l}$  ( $1 \times 10^6$  cells) of this suspension were placed in wells of 6 mm Linbro tissue culture plates (Flow Laboratories, Inglewood, Calif.) together with 100  $\mu\text{l}$  of serum supplemented RPMI. The mitogens used in this study were phytohaemagglutinin (PHA, Wellcome, Reagents, Ltd., Beckenham, England) and concanavalin A (Sigma) at concentrations of 25 and 50  $\mu\text{g/ml}$ . Mitogens were added in 20  $\mu\text{l}$  volumes to triplicate wells and unstimulated controls received 20  $\mu\text{l}$  of RPMI. The plates were mixed and incubated for 48 hr in a humidified atmosphere of 5% CO<sub>2</sub> in air, after which 20  $\mu\text{l}$  of tritiated thymidine (<sup>3</sup>H-T) (Thymidine-methyl-<sup>3</sup>H, New England Nuclear) containing 0.2  $\mu\text{Ci}$  was added to each well and the plates incubated for a further 18 hr. Harvesting was performed using a multiple automated sample harvester (MASH-II, Microbiological Associates, Bethesda, Md.). Incorporation of <sup>3</sup>H-T was assessed in a liquid scintillation spectrophotometer.

**Mitogen-induced protein synthesis.** In these studies 0.5-ml aliquots of cell suspensions ( $2 \times 10^6$  mononuclear cells) in serum supplemented RPMI were added to 17  $\times$  100 mm round bottomed polypropylene culture tubes (Falcon, Div. of Becton, Dickinson and Co., Oxnard, Calif.). The final volume in each tube was brought to 1

ml by the addition of 0.3 ml of medium, 0.1 ml of medium (controls), or 0.1 ml of PHA (experimentals) and 0.1 ml of a tritiated amino acid mixture ( $^3\text{H}$ -aa) containing 5  $\mu\text{Ci}$  (L-amino acid mixture, New England Nuclear) which was added at the outset. Two PHA concentrations were used, 25 and 50  $\mu\text{g}/\text{ml}$ , since these were previously found to induce maximal protein synthesis. The tubes were incubated for 72 hr at 37 C in a humidified atmosphere of 5%  $\text{CO}_2$  and air. After incubation 1 ml of 10% tricarboxylic acid was added to each tube. The resultant protein precipitate was twice washed with tricarboxylic acid and redissolved in 1 ml of 1 N KOH. Aliquots of 0.2 ml were transferred to 3 ml of acidified Insta-Gel (5.5 ml 17 N HCl per liter) and the extent of incorporation of  $^3\text{H}$ -aa into protein was assessed in a liquid scintillation spectrophotometer.

Using these techniques the preascorbate, during ascorbate, and postascorbate responses of neutrophils and lymphocytes were assessed. The leukocytes and sera from each individual were always matched for each time interval tested.

#### Serological studies

*Immunoglobulins and complement components.* The serum levels of IgG, IgA, and IgM were determined by radial immunodiffusion using commercial plates and standards (Behring Institute). Serum C'3 and C'4 complement levels were assayed by "rocket immunoelectrophoresis" (18, 19). Agarose gel plates (20  $\times$  10 cm) were used accommodating 32  $\times$  2.5 mm diameter wells. Serum samples were diluted 1/20 for both C'3 and C'4 estimations and applied in 5  $\mu\text{l}$  volume to each well. Dilutions of the serum standard were run with each plate. Antisera to human C'3 and C'4 were obtained commercially (Behring). Calibration curves were constructed by appropriate dilutions of standard sera. Electrophoresis was performed at 10 V for 3 hr. Results of immunoglobulin

and complement estimations are expressed as milligrams per 100 ml.

## Results

### Expression of results

The results of preascorbate testing are expressed as the mean and SE for the group (five individuals). Results obtained during ingestion of ascorbate and postascorbate are expressed as the mean stimulatory index with standard error for the group. Analysis of data was performed using Spearman's rank correlation coefficient. Preascorbate results for each test were compared with corresponding values obtained during and postascorbate ingestion.

### Neutrophil studies

Incubation of chambers was terminated at varying time intervals (1, 2, 3, 4, and 5 hr) and the in vitro kinetics of neutrophil movement to endotoxin activated autologous serum assessed. Table 1 indicates significant stimulation of cell motility at ascorbate doses of 2 and 3 g daily ( $P < 0.05$ ). The enhancement was evident in all individuals at each time interval tested.

The results of the studies on postphagocytic metabolic activity are shown in Table 2. There was no significant effect of ascorbate

TABLE 1  
The effects of increasing weekly doses of ascorbate on the kinetics of neutrophil chemotaxis to autologous EAS

Time hr	Preascorbate neutrophil chemotaxis (mean and SE) (cells per high power field)	Neutrophil chemotactic index (mean and SE) after:			
		1 g ascorbate	2 g ascorbate	3 g ascorbate	Postascorbate
1	5.2 $\pm$ 2.9	1.12 $\pm$ 0.40	3.11 $\pm$ 0.36	3.50 $\pm$ 0.94	0.73 $\pm$ 0.42
2	60.4 $\pm$ 8.8	1.06 $\pm$ 0.14	2.65 $\pm$ 0.41	2.94 $\pm$ 0.53	1.01 $\pm$ 0.25
3	80.0 $\pm$ 9.7	1.20 $\pm$ 0.21	2.89 $\pm$ 0.62	2.84 $\pm$ 0.51	1.14 $\pm$ 0.19
4	86.1 $\pm$ 10.6	0.95 $\pm$ 0.19	3.31 $\pm$ 0.43	3.08 $\pm$ 0.46	0.87 $\pm$ 0.23
5	70.2 $\pm$ 8.9	1.18 $\pm$ 0.25	3.67 $\pm$ 0.32	4.01 $\pm$ 0.58	1.07 $\pm$ 0.31

TABLE 2  
The effects of increasing weekly doses of ascorbate on neutrophil resting and postphagocytic hexose monophosphate shunt activity and protein iodination

	Preascorbate hexose monophosphate shunt ac- tivity (mean cpm and SE)	Index (mean and SE) of pre- and postphagocytic metabolic activity after:			
		1 g ascorbate	2 g ascorbate	3 g ascorbate	Postascorbate
Resting:	5,902 $\pm$ 675	1.15 $\pm$ 0.21	0.91 $\pm$ 0.19	0.94 $\pm$ 0.17	0.78 $\pm$ 0.20
Stimulated:	29,026 $\pm$ 1,068	1.08 $\pm$ 0.12	0.86 $\pm$ 0.22	1.10 $\pm$ 0.18	1.11 $\pm$ 0.19
Preascorbate protein iodination (mean cpm and SE)					
Resting:	4,941 $\pm$ 561	1.12 $\pm$ 0.18	0.80 $\pm$ 0.26	0.86 $\pm$ 0.16	0.91 $\pm$ 0.19
Stimulated:	24,092 $\pm$ 1,431	0.92 $\pm$ 0.19	0.85 $\pm$ 0.21	0.81 $\pm$ 0.22	0.89 $\pm$ 0.22

at any dose on the PMN resting and stimulated hexose monophosphate shunt activity. Similarly, myeloperoxidase mediated iodination of ingested *C. albicans* was unaffected.

#### Lymphocyte studies

Stimulation of lymphocyte transformation to both mitogens at both concentrations was observed after each dose of ascorbate (Table 3). This increase was significant for both mitogens after ingestion of 1, 2, or 3 g of ascorbate daily ( $P < 0.05$ ) for each mitogen concentration at each dose of ascorbate. There was no significant alteration in the extent of PHA-induced lymphocyte protein synthesis during ascorbate ingestion.

#### Serological studies

Levels of serum immunoglobulins and complement components showed no significant alteration during ascorbate ingestion (Table 4). Similarly, total hemolytic complement activity was unaffected (results not shown).

#### Discussion

Ingestion of ascorbate was found in the present study to promote stimulation of neu-

trophil chemotaxis to EAS. The enhanced motility was observed in each volunteer after the ingestion of 2 and 3 g of the vitamin daily and was evident at all time intervals tested in vitro. This would indicate that ascorbate, in appropriate doses, can hasten accumulation at early time intervals, increase the total number, and sustain the migration of PMN to a site of infection. These results are broadly in agreement with the findings of Boxer et al. (3) who observed stimulation of chemotaxis in the neutrophils of a patient with Chediak-Higashi syndrome after 200 mg of ascorbate daily and two normal individuals ingesting 1 g of ascorbate daily. We observed no effect on neutrophil motility after the ingestion of this dose of the vitamin. The fairly high dose required to stimulate PMN motility in vivo correlates well with the in vitro findings of Goetzl et al. (1). Similarly, we have also found that stimulation of neutrophil motility in vitro by ascorbate is dose dependent and requires fairly large amounts of the vitamin (R. Anderson, R. Oosthuizen, R. Maritz, A. Theron, and A. J. Van Rensburg, unpublished observations). A number of valid criticisms may be leveled at this component of the study—only one leukoattractant was used and no studies on random migration were

TABLE 3  
The effects of increasing weekly doses of ascorbate on mitogen-induced lymphocyte DNA and protein synthesis

	Preascorbate lymphocyte transformation (mean cpm and SE)	Index (mean and SE) of lymphocyte activation after:			
		1 g ascorbate	2 g ascorbate	3 g ascorbate	Postascorbate
Resting:	289 ± 47	1.21 ± 0.34	1.15 ± 0.29	1.21 ± 0.26	1.20 ± 0.19
25/μg PHA	17,828 ± 1,998	2.36 ± 0.51	2.61 ± 0.41	2.91 ± 0.35	1.35 ± 0.46
50/μg PHA	16,837 ± 2,306	2.81 ± 0.43	2.66 ± 0.50	2.54 ± 0.33	1.05 ± 0.49
25/μg Con A	2,549 ± 668	2.97 ± 0.56	3.03 ± 0.41	2.67 ± 0.41	1.21 ± 0.37
50/μg Con A	3,115 ± 703	3.91 ± 0.62	2.91 ± 0.36	3.10 ± 0.45	0.91 ± 0.32
Preascorbate lymphocyte protein synthesis (mean cpm and SE)					
Resting:	21,098 ± 2,793	1.20 ± 0.21	0.89 ± 0.24	0.95 ± 0.26	1.21 ± 0.25
25/μg PHA	43,198 ± 6,879	0.95 ± 0.19	1.16 ± 0.21	0.87 ± 0.23	1.16 ± 0.22
50/μg PHA	56,123 ± 7,218	1.16 ± 0.11	1.09 ± 0.20	0.91 ± 0.21	1.12 ± 0.21

TABLE 4  
The effects of increasing weekly doses of ascorbate on the concentrations of serum immunoglobulins and complement components

	Preascorbate serum C'3, C'4, and immunoglobulin levels (mean and SE) mg/100 ml	Serum immunoglobulin and C'3 and C'4 levels (mean and SE) after:			
		1 g ascorbate	2 g ascorbate	3 g ascorbate	Postascorbate
IgG	1,788 ± 72	1,664 ± 93	1,620 ± 85	1,688 ± 73	1,696 ± 81
IgA	262 ± 18	248 ± 19	240 ± 19	244 ± 18	242 ± 19
IgM	266 ± 48	264 ± 47	242 ± 48	250 ± 46	254 ± 47
C'3	52.3 ± 5.3	53.20 ± 5.2	60.80 ± 7.2	56.20 ± 5.7	61.00 ± 6.8
C'4	25.40 ± 2.8	25.60 ± 3.1	30.00 ± 5.1	27.60 ± 4.1	27.60 ± 3.5


performed; the small number of volunteers included in the study; no concomitant studies correlating intracellular ascorbate levels with enhanced motility were performed; no studies were performed using intermediate (e.g., 1.5 g daily) or broken (e.g.,  $3 \times 500$  mg daily) doses. However, due to the nature of the survey and the volumes of blood that would have been required, it was not possible to accommodate these studies. However, they could be included in a more restricted project to further investigate the effects of ingested vitamin C on PMN motility.

No effects of ascorbate ingestion on the extent of postphagocytic hexose monophosphate shunt activity or myeloperoxidase-mediated protein iodination were observed. No inhibition at "mega doses" of these activities was observed as previously reported by Shilotri and Bhat (8). This would indicate no *in vivo* effect of ascorbate on these activities although there are profound effects *in vitro* (4, 5).

Enhancement of lymphocyte transformation to PHA and Con A is in agreement with the results of Siegel and Morton (10) who reported 150 to 200% stimulation of Con A-induced DNA synthesis in the splenic T-lymphocytes of rats that had been fed a high ascorbate-containing diet. The enhanced mitogen responses observed in the present study were noted at all three doses of ascorbate and seemed to be of a similar magnitude. No effects on PHA-induced protein synthesis were observed. Siegel (11, 12) have reported that ascorbate can potentiate interferon production. The assay of protein synthesis used in the present study may be insensitive to enhanced lymphokine and interferon production that require more specialized assay techniques. Munster et al. (9) have reported *in vitro* inhibition of lymphocyte transformation to PHA by ascorbate at fairly high concentrations. We have also observed inhibition at high concentrations with occasional mild stimulation at lower concentrations (R. Anderson, R. Oosthuizen, R. Maritz, A. Theron, and A. J. Van Rensburg, unpublished observations).

Serum immunoglobulin levels, complement components, and total hemolytic complement activity remained unaltered by increasing doses of ascorbate. These observations are contradictory to those of Prinz et al. (13) who reported increased serum IgA, IgM,

and C'3 levels. This finding may be explained by the different durations of the two studies. However, Siegel and Morton (10) have reported no alteration in rat B-lymphocyte responses to bacterial endotoxin after ingestion of high dose ascorbate diets, which would seem to be in agreement with the present study.

There is disagreement as to the effects of vitamin C on immune function *in vivo* and resistance to disease. This may be due to an inadequate knowledge of what components of the immune system are affected by the vitamin and the dosages required to achieve stimulation. This report indicates that ascorbate stimulates certain cellular, but not humoral, immune functions in normal individuals. There is a differential stimulatory dose requirement for lymphocytes and neutrophils, the latter requiring more vitamin for enhancement of chemotactic responsiveness. This effect should be considered when interpreting the protective role of ascorbate in certain viral infections such as measles (20), influenza (21, 22), and recurrent herpes and warts (23) in which adequate PMN motility is probably of importance. 

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