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Vitamin C
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Lymphocytes
Concanavalin A
Cell-mediated Immunity
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Modulation of Certain Immunologic Responses by Vitamin C

II. Enhancement of Concanavalin A-Stimulated Lymphocyte Responses

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Summary: We have studied the potentiation of certain cellular immune responses in vitro and in vivo by vitamin C. Previously, we reported that vitamin C enhanced pokeweed mitogen-stimulated cultured human lymphocyte responses in vitro and, under certain circumstances, in vivo. We have extended this work by examining the effects of vitamin C upon in vivo and in vitro concanavalin A-stimulated lymphocyte responses. We found (1) vitamin C, at physiologic serum concentrations, significantly augmented concanavalin A-stimulated lymphocyte DNA synthesis in dose response fashion. (2) Optimal enhancement required pre-incubation of cells with vitamin C prior to mitogen stimulation as well as the addition of fresh vitamin C to culture daily, was (3) maximal in cells cultured for two days, and (4) related to continuous exposure of cells to fresh vitamin C throughout the culture. (5) Congeners of vitamin C (D-, dehydro, and sodium ascorbate) comparably enhanced lymphocyte responses. (6) In vitro enhancement was not a result of altered concanavalin A binding by lymphocytes. (7) In vitro concanavalin A-stimulated lymphocyte responses of individuals who had ingested 10 grams of vitamin C daily were comparable to those of a control group. These data confirm and broaden previous observations of the immuno-enhancing effects of vitamin C and provide a useful model for examining pharmacologic augmentation of lymphocyte responses.

Therapeutic augmentation of cellular immune responses in patients with depressed cell-mediated immunity would be expected to ameliorate clinical mani-

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festations of disease. Several pharmacologic agents have been utilized, with variable success, to modify abnormal immune responses [1, 3, 4, 5, 7, 8, 9, 14, 15] in patients. We have been interested in the effects of ascorbic acid upon *in vitro* and *in vivo* human lymphocyte responses. In previous studies we found ascorbic acid enhanced pokeweed mitogen-stimulated DNA and protein synthesis by human lymphocytes [11]. Herein we have extended these observations to the effects of ascorbic acid upon concanavalin A-stimulated lymphocytes.

Methods and Materials

Peripheral Blood Lymphocytes (PBL)

Blood from healthy subjects receiving no medications was collected and layered onto Ficoll-Hypaque, centrifuged, buffy coat at the interface removed and washed, as described [11]. Cells (reproducibly > 95 % viable mononuclear) were adjusted to 1×10^6/ml of culture. All studies were conducted after informed consent was obtained from volunteers (approved by institutional committees for the protection of human subjects).

Culture Techniques

RPMI 1640 medium (containing L-glutamine) (GIBCO, Grand Island, N. Y.) was used for cultures and was supplemented with 100 000 U penicillin, 250 µg amphotericin B, 100 000 µg streptomycin, 20 ml of 1 M HEPES buffer (GIBCO) and 1 % bovine serum albumin (BSA) (Sigma) per liter.

For most experiments, PBL were incubated overnight in sterile 12 × 75 mm culture tubes, at 1×10^6/ml in medium with or without 10 µg/ml ascorbic acid (Sigma). Cells were then centrifuged at 270 × G for 10 minutes, supernatant discarded, and resuspended in 1 ml of culture medium with or without 10 µg/ml ascorbic acid, and with or without 1 µg/ml concanavalin A (con A) (Sigma). This process was repeated after 24 hours of culture. Then 0.2 ml of the cell suspension was placed in wells of round-bottom microtiter plates (Linbro Chemical Company, New Haven, Conn.) and pulsed with 0.5 µCi/10 µl of methyl-^{3}H-thymidine (^{3}HTdR), specific activity 25 Ci/mM (Research Products International Corporation, Elk Grove Village, Ill.). They were harvested 18 hours later with a Skatron harvester (Flow Laboratories, Rockville, Md.) and counts per minute (cpm) determined as previously described [11].

Ascorbic Acid (AA) Preparation and Determination

Drug Preparation: L-ascorbic acid (Sigma), dehydro-ascorbic acid (ICN Pharmaceuticals, Cleveland, Ohio), and d-iso-ascorbic acid (Sigma) were obtained in powder form. Quantities of drug used for *in vitro* experiments were freshly weighed for each experiment and dissolved in complete medium. For *in vivo* experiments identically appearing vitamin C tablets or placebo tablets were kindly supplied by Dr. Myron Brin, Roche Research Institute, Nutley, N. J.

Sample Preparation. Plasma and leukocyte samples were collected, frozen, and stored for assay, as described [11].

Assay: Appropriate amounts of AA were weighed and dissolved in 5 % TCA to prepare standards. Samples were mixed with 2-4-dinitrophenylhydrazine reagent (Sigma), incubated, and the log of percentage of transmission plotted against the concentrations of the known standards, as described [11]. Concentrations of unknowns were determined from the standard curve.

Statistics

The arithmetic mean ($\bar{x}$), standard deviation of the mean, and standard error of the mean (SEM) were determined for quadruplicate samples. Results are expressed as $\bar{x} \pm$ SEM. Certain data were analyzed using t tests. For many experiments the percentage of enhancement of

3HTdR uptake by cells at various drug concentrations was calculated as percentage of enhancement

$$= \left[\frac{\text{cpm (stimulated cells in medium with drug)}}{\text{cpm (stimulated cells in medium without drug)}} - 1 \right] \times 100.$$

Results

Optimal Culture Conditions

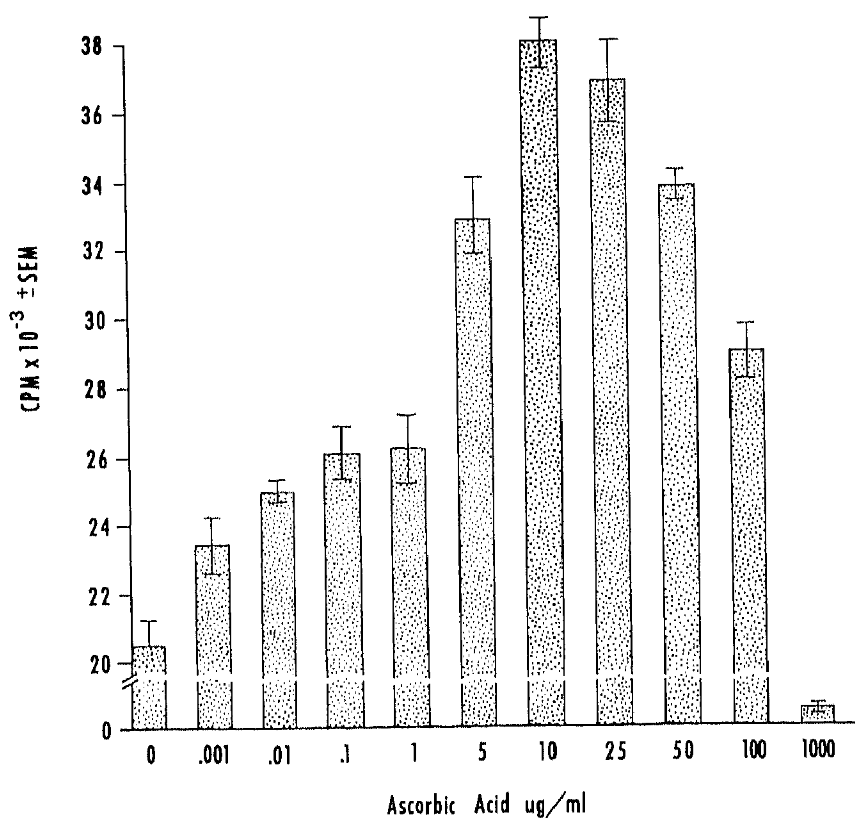
Our earlier studies indicated that optimal enhancement of PBL 3HTdR uptake by ascorbic acid in pokeweed mitogen-stimulated cultures occurred when lymphocytes were preincubated in ascorbic acid and the ascorbic acid was replenished daily [11]. We therefore examined con A-stimulated PBL responses under several culture conditions. (1) PBL not preincubated but cultured with con A displayed 117,490±1,177 cpm while cells not preincubated but cultured with both con A and vitamin C, showed 98,03 ± 4,039 (– 17 % change). (2) PBL preincubated 24 h in medium before culture with con A took up 95,579 ± 2,034 cpm while cells preincubated 24 h in 10 μg/ml AA followed by culture with con A 24 h later incorporated 92,292 ± 303 cpm (– 3 % change). (3) PBL not preincubated but cultured with con A and medium, changed daily, showed 72,659 ± 1,759 cpm while cells not preincubated but cultured with both con A and AA, changed daily, 117,987 ± 5,305 (+ 62 % change). (4) PBL preincubated 24 h in medium followed by culture with con A and medium, changed daily, had 40,476 ± 571 cpm whereas cells preincubated 24 h in AA and then cultured with AA and con A, changed daily, had 83,683 ± 1,156 cpm (+ 107 % change). Thus enhancement required replenishment of AA daily and was optimal when PBL were preincubated in AA before addition of mitogen. Data not shown indicate that optimal enhancement of PBL 3HTdR uptake by AA occurred after 48 hours of con A stimulation. Also not shown are observations that AA had no effect on unstimulated cells.

Optimal AA Concentration

After identifying the optimal culture conditions for AA enhancement of con A-stimulated lymphocytes, we examined the effects of various AA concentrations on PBL 3HTdR uptake. In the Figure a representative experiment illustrates that optimal enhancement occurred at physiologic serum concentrations of AA and that enhancement by AA was concentration-dependent.

Effects of Adding AA at Various Times during Culture

In these experiments, AA was added to appropriate cultures coincident with mitogen. Greatest enhancement (76 %) occurred when PBL were both preincubated in AA and AA was in culture for the entire culture period. Some enhancement (48 %) occurred when PBL were preincubated in AA and AA was in



Effect of various AA concentrations upon con A-stimulated PBL 3HTdR uptake. Cells were preincubated 24 h in medium or AA and then cultured with or without various concentrations of AA and with or without con A (1 µg/ml). Cells were harvested after 48 h with daily changes of AA and con A.

culture for only the last 24 hours. There was no statistically significant enhancement (7 %) when PBL were preincubated in AA only. In similar experiments, we examined the effect of removing AA from culture at 24 hour intervals. Enhancement of 3HTdR uptake increased with longer exposure to AA. Thus enhancement was related to continuous exposure of cells to fresh AA throughout culture.

Effects of Ascorbic Acid Congeners

To determine if enhancement was due to a nutritional need for the essential vitamin, we examined the effects of congeners of AA (D-, L-, dehydro-ascorbic acid and sodium ascorbate) upon con A-stimulated PBL 3HTdR uptake. All four congeners of ascorbic acid were capable of comparably enhancing 3HTdR uptake of con A-stimulated PBL. In five experiments, we found the following: con A-stimulated control culture, 18,765 ± 3,370 cpm; L-AA, 32,528 ± 4,352

(89 % enhancement); D-AA, 31,518 ± 3,944 (82 % enhancement); Na ascorbate, 29,400 ± 3,929 (69 % enhancement); and dehydro-AA, 29,112 ± 6,198 (66 % enhancement). The congeners had no effect on unstimulated cultures.

Effect of AA upon Con A Binding to PBL

One × 10^6 PBL/ml were cultured for 24 hours in the presence of 0.5 μg con A plus 0.5 μg 3H-con A (New England Nuclear, Boston, Mass.), with or without 10 μg/ml AA. PBL were then washed twice, resuspended, and transferred to scintillation vials. The amount of 3H-con A bound to PBL cell membranes was determined as previously described by our laboratory [2]. In three experiments con A binding did not increase significantly (4 ± 23 %). Enhancement of PBL 3HTdR uptake by AA was not due to increased con A binding.

Effects of Oral Ingestion of AA upon Human PBL Responses in a Double-Blind, Placebo-Controlled Study

To determine whether oral ingestion of AA by normal volunteers would result in enhancement of responses of their lymphocytes when stimulated by con A *in vitro,* 16 volunteers were given either placebo or 10 g of vitamin C daily for one week. Individuals were bled before and within 3 hours after their last dose of vitamin C. *In vitro* con A-stimulated PBL 3HTdR uptake was determined for cells isolated from each bleeding. Oral ingestion of 10 g vitamin C daily had no effects on *in vitro* con A-stimulated 3HTdR uptake by PBL as shown in the table. Plasma and leukocyte intracellular levels of AA were also measured. Seven days of oral ingestion of AA did not produce any significant change in intracellular levels. Plasma levels of vitamin C were significantly ($p < 0.0005$) increased in the vitamin C-treated group. No correlations were noted between plasma levels and 3 HTdR uptake.

Discussion

A number of rheumatic, infectious, neoplastic, and immunologic diseases as well as the phenomena of aging and malnutrition [5, 6, 9, 13, 15] have been characterized by impaired cellular immune mechanisms. Among therapeutic approaches to these disorders is the hope that manipulation of abnormal immune responses may render them normal. Only recently has attention been directed to the potential of pharmacologic agents to selectively alter immune responses. Restoration of immunoregulatory balance in disease has proved clinically efficacious in both animal models and certain human diseases. Transfer factor, thymosin, soluble immune response suppressor substances, levamisol, and bacillus Calmette-Guérin have been studied in immunologic disease with instances of clinical improvement [1, 3, 4, 5, 7, 8, 9, 12, 14, 15].

Tab. 1: Effects of Oral Ingestion of AA upon in vitro Con A-Stimulated PBL 3HTdR Uptake and Plasma and Leukocyte AA Levels

	Before				After			
	CPM	TI *	Plasma µg/ml	WBC µg/5×10⁶	CPM	TI *	Plasma µg/ml	WBC µg/5×10⁶
Vitamin C Group								
CC	52,108	7.9	8.25	1.04	18,481	7.8	12.10	1.37
PC	40,420	15.6	8.20	1.50	37,856	13.7	14.55	1.78
DY	21,051	3.6	7.89	1.54	22,178	2.1	31.92	1.88
RP	44,231	5.7	13.75	1.81	36,940	6.5	17.07	1.15
AL	35,682	8.0	6.71	1.56	14,422	3.4	23.38	1.62
WC	31,902	6.5	7.57	1.19	25,509	9.03	19.80	1.57
PM	65,314	4.9	4.57	2.90	46,735	4.6	15.81	2.22
JW	24,493	8.4	6.97	1.41	6,049	3.0	12.3	1.51
KR	41,656	11.5	5.99	1.21	27,284	8.9	21.97	1.57
x̄ ± SEM ...	36,317 ± 5,927	8.0 ± 1.2	7.77 ± 0.84	1.57 ± 0.18	26,161 ± 4,241	6.6 ± 1.3	18.77 ± 2.11	1.63 ± 0.10
Placebo Group								
JL	32,473	5.2	7.27	1.82	12,654	1.6	5.23	1.38
SL	30,996	7.9	3.33	2.22	27,964	5.4	5.91	1.76
BA	35,038	4.0	4.64	1.40	12,449	6.9	5.09	1.56
AD	26,399	13.5	5.84	1.33	33,582	5.9	10.21	1.59
JG	47,709	5.4	8.94	1.69	50,848	6.4	10.18	1.49
ME	46,323	5.3	6.03	1.92	39,834	7.9	7.28	2.06
CP	30,481	8.6	4.70	1.17	10,351	3.3	4.95	1.09
x̄ ± SEM ...	35,631 ± 3,107	7.7 ± 1.5	5.83 ± 0.70	1.65 ± 0.14	26,811 ± 5,920	5.3 ± 0.8	6.98 ± 0.88	1.56 ± 0.11

* Volunteers ingested 10 g of AA or placebo daily for 7 days. *In vitro* PBL uptake of 3HTdR was determined before study and immediately after the last dose of AA. One × 10^6 PBL/ml were incubated overnight in 1 % BSA in RPMI, supernatant discarded, fresh medium then added with con A (1 µg/ml), and cultured for 24 h. Medium and con A were replenished once more before harvesting at 48 h. Cpm and transformation indices (TI) were determined.

There has been much interest in the possibility that ascorbic acid may enhance host resistance [3, 11, 12]. These observations have been reviewed elsewhere and are beyond the scope of this discussion. We have therefore utilized *in vitro* human lymphocyte 3HTdR incorporation to cellular DNA, a well-recognized *in vitro* correlate of *in vivo* cell-mediated immunity, as a model in which to examine pharmacologic enhancement of ascorbic acid [11].

In previous studies we reported that vitamin C enhanced pokeweed mitogen-stimulated cultured human lymphocyte responses *in vitro* and, under certain circumstances, *in vivo* [11]. The present work has confirmed and extended these studies. We have found: (1) vitamin C, at physiologic serum concentrations, significantly augmented con A-stimulated lymphocyte DNA synthesis in dose-response fashion. (2) Optimal enhancement required preincubation of cells

with vitamin C prior to mitogen stimulation, as well as the addition of fresh vitamin C to culture daily, was (3) maximal in cells cultured for two days, and (4) related to continuous exposure of cells to fresh vitamin C throughout the culture. (5) Congeners of vitamin C (d-, dehydro-, and sodium ascorbate) comparably enhanced lymphocyte responses. (6) *In vitro* enhancement was not a result of altered con A-binding by lymphocytes. (7) *In vitro* con A-stimulated lymphocyte responses of individuals who had ingested 10 g of vitamin C daily were comparable to those of a control group.

We have previously considered whether these *in vitro* effects of vitamin C indeed reflect pharmacologic augmentation of immunologic responses or merely a nutritional need for vitamin C in cell culture. For all the reasons reviewed [11] plus our observations that congeners of L-ascorbic acid were equally effective immunoenhancing agents, it appears that the effects are pharmacologic. The findings in the present study are quite similar in most respects to those previously reported. Optimal culture conditions were similar in that both preincubation and daily replenishment of medium were necessary. Also, optimal enhancement related to duration of exposure of cells to vitamin C in culture. However, the kinetics of the optimal responses to these two agents differed. This is consistent with data that pokeweed mitogen and concanavalin A exhibit specificities for overlapping but different populations of lymphocytes. That vitamin C augmented responses to both of these mitogens indicates a broad spectrum of action on diverse human lymphocyte subpopulations. The failure of oral ingestion of 10 grams of vitamin C to augment cultured lymphocyte con A-stimulated responses, as compared to placebo controls, was unexpected and differs from our earlier observations. However, slight differences in the design of the studies may explain the disparities – use of different mitogens, and therefore presumably examination of different cell subpopulations; differing quantities of vitamin C; and study of the volunteers at slightly different times. It appears that only under certain experimental conditions can vitamin C enhance immune responses *in vivo*. Further work is warranted to carefully examine these circumstances and better define the mechanism of action of this unique effect of vitamin C.

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References

1. ANSELL, B. (Chairman): J. Rheumatology. *4* (Suppl.), 1 (1978). – 2. ANTHONY, C. R., PANUSH, R. S.: Clin. Exper. Immunol. *31*, 482 (1978). – 3. BOXER, L. A., WATANABE, A. M., RISTER, M., BESCH, H. R., ALLEN, J., BAEHNER, R. L.: N. Engl. J. Med. *295*, 1041 (1976). – 4. CAMERON, E., PAULING, L.: Proc. Nat. Acad. Sci. 72, 3685 (1976). – 5. GERSHWIN, M. E., AHMED, A., STEINBERG,

A. D., THURMAN, G. B., GOLDSTEIN, A. C.: J. Immunol. *113*, 1068 (1974). – 6. HALLGREN, H. M., YUNIS, E. J.: J. Immunol. *118*, 2004 (1977). – 7. HITZIG, W. H., GROB, P. J.: Prog. Clin. Immunol. 2, 69 (1974). – 8. JORDAN, G. W., MERIGAN, T.: Annu. Rev. Pharm. *15*, 157 (1975). – 9. KRAKAUER R. S., STROBER, W., RIPPEON, D. L., WALDMANN, T. A.: Science *196*, 56 (1977). – 10. LAW, D. K., DUDRICK, S. J., ABDOU, N.I.: Ann. Intern. Med. *79*, 545 (1973). – 11. PANUSH, R. S., DELAFUENTE, J. C.: Internat. J. Vit. Nutr. Res., Supplement No. 19, p. 179 (1979). – 12. PAULING, L.: San Francisco, W. H. Freeman and Co. (1970). – 13. STOBO, J. D., PAUL, S., VAN SCOY, R. E., HERMANS, P. E.: J. Clin. Invest. *57*, 319 (1976). – 14. SUTHERLAND, R. M.: J. Rheum. *1*, 349 (1974). – 15. WALDMANN, T. A.: Ann. Intern. Med. *88*, 226 (1978).

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