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The Effect of Ascorbic Acid Supplementation on some Parameters of the Human Immunological Defence System

W. PRINZ, R. BORTZ, B. BREGIN and M. HERSCH

Department of Physiological Chemistry, University of the Witwatersrand, Medical School,
Johannesburg

Summary: We have investigated the effect of ascorbic acid (vitamin C) supplementation on some parameters of the human immune defence system in a group of 25 healthy, male university students. The subjects ingested 1 g ascorbic acid per day for a period of 75 days. Serum levels of IgA, IgG, IgM, C-3 complement component, cortisol and transcortin were measured before and after the ascorbic acid course. Corresponding measurements were performed on a control group of 20 healthy, male university students receiving no extra-dietary vitamin C.

Our results showed that ascorbic acid supplementation caused a statistically significant increase in the serum levels of IgA, IgM and C-3 complement. Our study does not permit of conclusions regarding the mechanisms of action of ascorbic acid.

Introduction

The reputed virtues of ascorbic acid in optimizing health in general and resistance to infectious diseases in particular [17] remain controversial [13]. Physiological effects ascribed to vitamin C are multifarious [cf. 14].

The best known and most extensive clinical trials with ascorbic acid were concerned with the common cold. Essentially, the question asked was whether or not ascorbic acid prevented and/or ameliorated the symptoms of this viral infection. Reviews of these studies and evaluations of the evidence presented have recently appeared [4, 18, 23]. There was no concensus of opinions. On balance, statistical analysis appear to be in favour of vitamin C. However, clinical

trials of this nature are probably more qualitative than quantitative. It is impossible to ascertain whether persons taking extradietary doses of vitamin C should have caught a cold if they had not taken this vitamin. As to a possible ameliorating or attenuating effect of vitamin C, the assignment of degrees of severity of a cold is clearly a matter of subjective opinion.

If one accepts PAULING's hypothesis regarding the beneficence of vitamin C [8, 16, 17] it should be possible to obtain quantitative data in support thereof. In the present study, we endeavoured to ascertain whether ascorbic acid supplementation had any directly quantifiable effect on some parameters of the immunological defence system. Specifically, the parameters chosen were the serum levels of the three main antibodies (IgA, IgG, IgM), C-3 complement, cortisol and transcortin.

The reason for investigating serum Ig-levels is self-evident. The choice of C-3 in preference to any of the other complement fractions is justified on several grounds. It is quantitatively the most prominent complement fraction in serum. It is also the key component at the juncture of both the "classical" and the "alternate" pathways of the complement-mediated defence mechanisms. Furthermore, C-3 appears to play a key role in the triggering of B-cells toward antibody formation [6, 9] as well as in the enhancement of phagocytosis [1, 20]. The effect of cortisol in suppressing the immune response is well-known. The inclusion of transcortin in our study was prompted by the consideration that cortisol is physiologically active only in the unbound form.

Methods and Materials

The subjects for our study were 45 volunteer male medical students aged between 18 and 21. Sole criteria for the selection of the volunteers were that they should be "normal" and "healthy", nonvegetarians and not previously exposed to extradietary doses of vitamin C. The subjects were divided into a "control" group of 20 students and an "experimental" group of 25 students¹.

The test-period extended over 11 weeks, during which all participants continued on their normal diets. Members of the "experimental" group were required to ingest 1 g vitamin C (Cebion, Merck) per day. Blood was drawn from all participants before commencing with ascorbic acid supplementation of the diet and again at the end of the 11-week test period. The time of bleeding was set at between 9 *am* and 10 *am* on both occasions, so as to correspond with peak values in the circadian rhythm of serum cortisol concentrations [cf. 15]. Sera were preserved with NaN₃ (1 mg/ml) and stored deep-frozen until required for the assays. Sera obtained from all 45 participants at the end of the test period were assigned a random-number code, so as to eliminate investigator bias. Decoding was effected only after all the assays were completed.

Serum levels of IgA, IgG and IgM were determined by radial immunodiffusion, C-3 and transcortin concentrations were quantified by "rocket immunoelectrophoresis". Cortisol assays were performed using a Radio Immuno Assay/Competitive Protein Binding technique.

¹ The "experimental" group was purposely made larger than the "control" group in anticipation of several drop-outs from the former group. In the event, this contingency planning proved unnecessary.

Tab. Ia: Serum Immunoglobulin Levels (I. U./ml)
IgA

Subject	Control			Experimental		
	1st Bleeding	2nd Bleeding	Change	1st Bleeding	2nd Bleeding	Change
1	162	123	- 39	188	202	+ 14
2	98	82	- 16	210	235	+ 25
3	158	130	- 28	120	102	- 18
4	105	91	- 14	195	210	+ 15
5	68	64	- 4	140	150	+ 10
6	97	80	- 17	130	107	- 23
7	92	61	- 31	57	67	+ 10
8	88	84	- 4	71	78	+ 7
9	133	188	- 15	72	52	- 20
10	193	155	- 38	93	100	+ 7
11	68	45	- 23	240	195	- 45
12	187	210	+ 23	74	75	+ 1
13	80	84	+ 4	220	258	+ 38
14	97	69	- 28	135	195	+ 60
15	57	58	+ 1	105	104	- 1
16	127	130	+ 3	65	78	+ 13
17	158	150	- 8	187	155	- 32
18	111	75	- 36	160	155	- 5
19	100	66	- 34	94	120	+ 26
20	107	130	+ 23	180	160	- 20
21				97	84	- 13
22				62	68	+ 6
23				37	50	+ 13
24				140	118	- 22
25				78	100	+ 22
Mean:	114.3	100.2	- 14.1	126.0	128.7	+ 2.7
S. D.:	39.2	41.9	18.7	57.9	59.4	23.4
		P < 0.005			P. N. S.	

1. Serum Immunoglobulin Levels

Materials: Anti-IgA serum, specific for α -chains (Dako cat. no. 10-MAT). Anti-IgG serum, specific for γ -chains (Dako cat. no. 10-090). Anti-IgM serum, specific for μ -chains (Dako cat. no. 10-091). Reference standard: *Behring Institute* Standard Human Serum. Agar, purified (*Behring*).

Method: The serum levels of IgA, IgG and IgM were determined by the "radial immunodiffusion" (R. I. D.) technique of HOBBS [10] incorporating the modifications suggested by SHULMAN [21]. Agar gels, 1.5 mm in depth and incorporating the appropriate anti-serum, were prepared in Petri dishes. 2 mm Diameter wells were cut and promptly filled with appropriately diluted serum samples². For the construction of calibration curves, 4 serial dilutions of the "standard" serum were included on each gel. Radial immunodiffusion was allowed to proceed

² The appropriate dilutions employed were 1/15, 1/7 and undiluted serum for IgG, IgA and IgM respectively. The usual serum diluent for R. I. D. assays (0.14 M saline) was used.

Tab. 1b: Serum Immunoglobulin Levels (I. U./ml)
IgG

Subject	Control			Experimental		
	1st Bleeding	2nd Bleeding	Change	1st Bleeding	2nd Bleeding	Change
1	160	142	- 18	182	125	- 57
2	145	74	- 71	118	145	+ 27
3	205	142	- 63	88	142	+ 54
4	157	170	+ 13	121	118	- 3
5	140	144	+ 4	70	71	+ 1
6	133	139	+ 6	76	130	+ 54
7	167	121	- 46	62	47	- 15
8	124	108	- 16	106	106	+ 0
9	135	159	+ 24	88	130	+ 42
10	110	145	+ 35	81	79	- 2
11	108	139	+ 31	100	142	+ 42
12	106	130	+ 24	121	160	+ 39
13	74	64	- 10	118	90	- 28
14	76	98	+ 22	70	86	+ 16
15	122	90	- 32	100	72	- 28
16	108	108	+ 0	84	84	+ 0
17	88	140	+ 52	74	132	+ 58
18	91	132	+ 41	70	130	+ 60
19	60	98	+ 38	70	70	+ 0
20	100	100	+ 0	121	157	+ 36
21				81	130	+ 49
22				76	108	+ 32
23				66	72	+ 6
24				114	130	+ 16
25				91	80	- 11
Mean:	120.4	122.1	+ 1.7	93.9	109.4	+ 15.5
S. D.:	35.8	28.4	34.6	26.9	31.8	31.1
		P. N. S.			P < 0.02 (N. S.)	

at room temperature for 18 hours (IgA and IgG) or 22 hours (IgM). Precipitin ring diameters were measured using a viewing box and ruler (Partigen, Behring). Assays were performed in duplicate and reproducibility was checked on separate gels. A $\pm 4\%$ tolerance was allowed between the readings of duplicate assays. Results were expressed in I. U./ml as recommended by the W. H. O. [19] see Tables Ia - Ic.

2. Serum C-3 Complement levels

Materials: Anti-C-3 serum (Dako cat. no. 10-062). Reference "standard": Behring Institute standard Human Serum. Agarose (Miles Laboratories).

Method: Serum C-3 complement levels were assayed by "rocket immunoelectrophoresis" [2, 12]. The working procedure adopted was that described by WEEKE [2, chap. 2] and outlined in the Dako manual on "Working Procedures for Rocket Immunoelectrophoresis and Single Radial Immunodiffusion". 20×10 cm Agarose gel plates were employed accomodating 32 2.5 mm diameter wells. 5 μ l Aliquots of diluted serum samples (or "standard" serum dilutions) were pipetted into each well. All serum samples were diluted 1 + 20 with gel buffer. Four serial

Tab. 1c: Serum Immunoglobulin Levels (I. U./ml)
IgM

Subject	Control			Experimental		
	1st Bleeding	2nd Bleeding	Change	1st Bleeding	2nd Bleeding	Change
1	175	145	- 30	230	290	+ 60
2	230	240	+ 10	260	260	+ 0
3	230	165	- 65	230	191	- 39
4	165	170	+ 5	260	280	+ 20
5	119	119	+ 0	133	170	+ 37
6	160	185	+ 25	130	191	+ 61
7	200	112	- 88	239	290	+ 51
8	250	255	+ 5	159	200	+ 41
9	185	140	- 45	190	145	- 45
10	164	110	- 54	80	105	+ 25
11	190	165	- 25	190	224	+ 34
12	181	189	+ 8	242	201	- 41
13	150	210	+ 60	190	230	+ 40
14	131	142	+ 11	124	140	+ 16
15	120	140	+ 20	270	275	+ 5
16	250	250	+ 0	80	140	+ 60
17	210	210	+ 0	218	245	+ 27
18	180	155	- 25	88	96	+ 8
19	152	103	- 49	133	205	+ 72
20	240	260	+ 20	175	211	+ 36
21				175	185	+ 10
22				175	140	- 35
23				107	175	+ 68
24				80	74	- 6
25				238	320	+ 82
Mean:	184.1	173.2	- 10.9	175.8	199.3	+ 23.5
S. D.:	40.9	50.1	35.9	62.0	64.9	36.5
		P. N. S.			P < 0.005	

dilutions of the "standard" serum - ranging from 1 + 10 to 1 + 40 - were included, in duplicate, on each plate. These were used to construct calibration curves. A "control" serum (diluted 1 + 20) was also run in duplicate on each gel to ensure reproducibility in day-to-day runs [cf. 2]. Electrophoresis was performed at 10 V per cm for 3 hours. Rocket heights varied from 12 to 36 mm. All assays were performed in duplicate. Reproducibility was checked by running each member of a duplicate pair on separate gels. The accepted margin of tolerance in the values obtained for members of a duplicate pair was $\pm 4\%$. Results were expressed in mg/100 ml - see Table II.

3. Serum Cortisol Levels

Materials: "Cortipac" Kits for cortisol C. P. B. assay (the *Radiochemical Centre*, Amersham, England; Code SC.6).

Method: Total serum cortisol levels were assayed by a competitive protein binding technique, utilizing ^{75}Se -cortisol [3]. For these assays, the commercially available kits (*Radiochemical Centre*, Amersham, England) were employed, following the manufacturers' instructions.

Tab. II: Serum C-3 Complement Levels (mg/100 ml)

Subject	Control			Experimental		
	1st Bleeding	2nd Bleeding	Change	1st Bleeding	2nd Bleeding	Change
1	76	77	+ 1	91	92	+ 1
2	99	103	+ 4	82	91	+ 9
3	99	91	- 8	77	89	+ 12
4	87	114	+ 27	94	143	+ 49
5	99	84	- 15	91	92	+ 1
6	153	118	- 35	101	161	+ 60
7	92	94	+ 2	99	106	+ 7
8	121	86	- 35	79	96	+ 17
9	94	82	- 12	81	77	- 4
10	113	91	- 22	89	94	+ 5
11	129	91	- 38	96	92	- 4
12	113	99	- 14	86	113	+ 27
13	94	77	- 17	104	103	- 1
14	106	74	- 32	77	99	+ 22
15	72	84	+ 12	96	121	+ 25
16	96	74	- 22	97	121	+ 24
17	99	129	+ 30	96	114	+ 18
18	76	82	+ 6	86	101	+ 15
19	94	94	+ 0	91	94	+ 3
20	109	94	- 15	84	79	- 5
21				77	71	- 6
22				82	89	+ 7
23				72	91	+ 19
24				109	108	- 1
25				99	121	+ 22
Mean:	101.1	91.9	- 9.2	89.4	102.3	+ 12.9
S.D.:	19.0	14.8	19.5	9.7	20.1	16.3
		P < 0.05 (N. S.)			P < 0.001	

γ -counting was performed in a *Packard* Auto-Gamma Scintillation Spectrometer for 2 mins. (40 000-60 000 counts). Assays were performed in duplicate, allowing for a maximum margin of tolerance of $\pm 4\%$ in recorded counts. A calibration curve was obtained with 4 reference "standards", supplied by the manufacturers of the kits. Results were expressed as $\mu\text{g}/100\text{ ml}$ - see Table III.

4. Serum Transcortin Levels

Materials: Anti-serum against human transcortin: such an anti-serum is not yet commercially available. It was obtained as a gift from Dr. H. van Baelen³.

Transcortin standard: serum pool from 25 normal donors. In a dilution of 1+3 with gel buffer it was assigned a concentration value of 100 arbitrary units (A. U.) transcortin. Agarose (*Miles Laboratories*).

³ Dr. H. van Baelen, Rega-Instituut, Laboratorium voor Experimentele Geneeskunde, Minderbroederstraat 10, 3000 *Leuven*, *Belgium*.

Tab. III: Serum Cortisol Levels ($\mu\text{g}/100\text{ ml}$)

Subject	Control			Experimental		
	1st Bleeding	2nd Bleeding	Change	1st Bleeding	2nd Bleeding	Change
1	8.7	11.8	+ 3.1	11.4	18.8	+ 7.4
2	20.5	14.6	- 5.9	6.9	7.8	+ 0.9
3	9.4	9.8	+ 0.4	15.2	9.8	- 5.4
4	12.4	12.0	- 0.4	10.4	13.3	+ 2.9
5	14.2	13.4	- 0.8	11.4	14.0	+ 2.6
6	16.8	9.8	- 7.0	11.0	11.4	+ 0.4
7	16.5	10.5	- 6.0	19.5	17.0	- 2.5
8	10.7	11.4	+ 0.7	18.0	14.3	- 3.7
9	12.0	9.3	- 2.7	13.6	17.0	+ 3.4
10	6.1	7.9	+ 1.8	8.3	9.1	+ 0.8
11	21.0	22.0	+ 1.0	11.2	14.0	+ 2.8
12	17.0	13.8	- 3.2	10.6	11.7	+ 1.1
13	13.5	11.4	- 2.1	10.3	10.2	- 0.1
14	14.8	15.3	+ 0.5	10.8	10.4	- 0.4
15	20.2	12.4	- 7.8	10.6	8.6	- 2.0
16	8.7	10.2	+ 1.5	9.8	11.8	+ 2.0
17	14.8	10.6	- 4.2	16.4	15.0	- 1.4
18	11.0	11.0	0	10.6	9.4	- 1.2
19	20.0	19.8	- 0.2	18.0	7.1	- 10.9
20	17.5	12.8	- 4.7	14.3	11.0	- 3.3
21				11.7	12.6	+ 0.9
22				13.0	11.4	- 1.6
23				11.0	7.4	- 3.6
24				17.0	16.8	- 0.2
25				15.8	14.3	- 1.5
Mean:	14.3	12.5	- 1.8	12.7	12.2	- 0.5
S. D.:	4.4	3.4	3.2	3.3	3.2	3.5
			P < 0.03 (N. S.)			P. N. S.

Method: Serum transcortin levels were determined by a rocket immunoelectrophoresis technique as described under 2. The customary barbitone buffer, pH 8.6 was employed.

In the preparation of the agarose gel, an anti-serum concentration of 20 μl per 30 ml gel buffer was found to give optimal results. 2.5 Diameter wells were punched into the gel. Sera were diluted 10 + 75 with buffer and 5 μl aliquots applied per well. For the construction of the calibration curve, 3 dilutions of "standard" serum were employed, corresponding to 66, 50 and 25 A. U. This range was large enough to cover all actual serum values obtained. A "control" serum was included on all plates to check reproducibility in day-to-day runs. Rocket heights varied from 16-42 mm. All assays were performed in duplicate, allowing for a $\pm 4\%$ margin of error. Results were expressed as Arbitrary Units - see Table IV.

Statistics

Results obtained were analyzed using Student's t-test for Paired Samples. P-values < 0.01 were regarded as statistically significant. Means, Standard Deviations and P-values are recorded in Tables I-IV.

Tab. IV: Serum Transcortin Levels (Arbitrary Units)

Subject	Control			Experimental		
	1st Bleeding	2nd Bleeding	Change	1st Bleeding	2nd Bleeding	Change
1	44.5	47.5	+ 3	59	52.5	- 6.5
2	50	51	+ 1	44	41.5	- 2.5
3	37.5	37.5	0	58	58	0
4	55	50	- 5	55	53	- 2
5	53	49	- 4	58	56	- 2
6	55.5	48	- 7.5	48	48.5	- 0.5
7	56	51.5	- 4.5	50	57.5	+ 7.5
8	44.5	35	- 9.5	50	49	- 1.0
9	49.5	47	- 2.5	47.5	44.5	- 3
10	44.5	47.5	+ 3	55.5	50.5	- 5
11	44.5	52.5	+ 8	50	51	+ 1
12	49.5	45	- 4.5	51	54	+ 3
13	36.5	37	+ 0.5	44.5	52	+ 7.5
14	44.5	54.5	+ 10	45.5	48	+ 2.5
15	42	49	+ 7	44.5	47	+ 2.5
16	54.5	46.5	- 8	53	62.5	+ 9.5
17	50	52.5	+ 2.5	51.5	60.5	+ 9
18	41.5	46.5	+ 5	38.5	44.5	+ 6
19	62.5	61	- 1.5	44	46.5	+ 2.5
20	40.5	41.5	+ 1	44.5	44	- 0.5
21				49	52.5	+ 3.5
22				49	48	- 1.0
23				49	51.5	+ 2.5
24				43	48	+ 5
25				60.5	68.5	+ 8
Mean:	47.8	47.5	- 0.3	49.7	51.5	+ 1.8
S. D.:	6.8	6.2	5.4	5.65	6.3	4.4
		P. N. S.			P > 0.05 (N. S.)	

Results and Discussion

Serum IgA levels are reproduced in Table Ia. There was no significant change in the serum IgA levels of the "Experimental" group. The "control" group, on the other hand, exhibited a statistically significant ($P < 0.005$) 15% decrease in their serum levels of IgA. This result was somewhat unexpected and is difficult to interpret. On the statistical evidence, it is probably permissible to argue that the serum IgA levels of the "Experimental" group should similarly have decreased by 15% had they not been on a vitamin C-supplemented diet. It is relevant to recall that IgA is the principal immunoglobulin in exocrine secretions. A large proportion of the amount of IgA synthesized daily is lost in the mucous secretions [cf. 7].

Conceivably, the secretory drain on IgA may be subject to seasonal variations. Our study was, in fact, begun in early winter (1st bleeding on the 2nd July—winter in our latitude) and terminated at the beginning of spring (2nd bleeding on the 15th September). It is implicit in this reasoning that ascorbic acid does, in some way, counteract a potential net decrease in serum IgA levels during the winter months.

In the case of serum IgG levels (Table Ib) there was no significant change in the "control" group. "Experimental" group IgG levels showed a mean increase of 15% but the degree of statistical significance ($P < 0.02$) was not considered to be satisfactory.

Serum IgM levels (Table Ic) of the "control" group decreased non-significantly by some 8%. In contrast, the mean increase in IgM levels of the "experimental" group was found to be statistically significant ($P < 0.005$). The mean increase relative to that of the "control" group was approximately 20%. It is worthy of mention that IgM is the only class of antibody circulating in peripheral blood at concentrations higher than in tissues [11]. Furthermore, it is the first class of antibody to be produced upon infection, *ie*: antigenic stimulation [22]. The current view is that the immune system is especially geared to react promptly to non-specific infections by IgM secretion from spleen B cells [5]. Our study indicates that ascorbic acid has an enhancing effect on this immunological defence reaction.

The serum C-3 levels (Table II) of the "control" group, were, on average, decreased by some 10% during the test-period. However, this change was not statistically significant ($P < 0.05$). In contrast, the "experimental" group showed a mean increase in serum C-3 levels of about 15%. This increase was found to be statistically highly significant ($P < 0.001$).

The C-3 complement component has been shown to have a direct effect on the mobilization of leukocytes and the rate of phagocytosis of foreign cells [1, 20]. Our results, therefore, suggest that vitamin C should have an enhancing effect on this process by raising serum C-3 levels.

Our finding that ascorbic acid appears to raise the serum levels of both IgM and C-3 highlights an apparent reciprocal relationship between IgM and C-3. Both are efficient opsonins. Moreover, IgM is also a powerful activator of complement (*cf.* 7), whereas C-3 has been directly implicated in the triggering of B-cells [6, 9], the initial products of which would be IgM antibodies.

The serum cortisol levels of the "control" and "experimental" groups are summarized in Table III. The mean values obtained are in agreement with expectations for the time of day when blood was drawn. However, neither group showed any significant change in serum cortisol levels.

The results of our serum transcortin assays are recorded in Table IV. The mean value of serum transcortin levels of the "control" group suffered virtually

no change over the test-period. The corresponding parameter for the "experimental" group increased slightly, but statistically non-significantly ($P > 0,05$).

As mentioned previously, total serum cortisol levels should be considered in conjunction with serum transcortin levels. Our studies indicate that neither are affected by vitamin C. It follows, therefore, that ascorbic acid does not effect serum levels of physiologically active, free (*ie.*: unbound) cortisol.

In summary, we have found that ascorbic acid (vitamin C) supplementation raises the serum levels of IgA, IgM and complement component C-3. However, our experimental studies do not permit of any conclusion regarding the mechanism whereby ascorbic acid may effect these parameters.

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Dr. W. Prinz, Department of Physiological Chemistry, University of the Witwatersrand, Medical School, Hospital Street, Johannesburg, South Africa