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– supplementation
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– Ig M-type antibodies

Immune Response
– antigen induced stimulation
Sheep Red Blood Cells
Guinea Pigs

A Systematic Study of the Effect of Vitamin C Supplementation on the Humoral Immune Response in Ascorbate-Dependent Mammals

I. The Antibody Response to Sheep Red Blood Cells (a T-dependent antigen) in Guinea Pigs

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Summary: We have investigated the humoral immune response to Sheep Red Blood Cells (S.R.B.C. – a T-dependent cellular antigen) in two groups of random-bred guinea pigs. A control group of animals was maintained on a «minimal» ascorbate intake, while the test group animals received supplementary vitamin C in their drinking water. Serum haemolytic antibody levels were monitored at various intervals subsequent to immunization with S.R.B.C. The primary antibody response of the ascorbate-supplemented animals differed statistically significantly from that of the controls in two respects. The peak antibody titre was both higher and occurred earlier in the test animals. No significant difference in the antibody response to a secondary injection of antigen was manifest. Our observations suggest that vitamin C stimulates the elaboration of Ig M-type antibodies in particular.

Introduction

Studies on the involvement of vitamin C in a variety of immunological phenomena have recently been reviewed [11]. There have been few published reports on the possible role of ascorbate in the humoral immune response. Thus far, the evidence implicating vitamin C in an enhanced *in vivo* synthesis of antigen-specific antibodies has been inconclusive [6, 7]. A major defect has

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been the deployment of insufficiently precise methods, such as haemagglutination [6], for the quantitation of antigen-specific antibodies.

In a previous study [10] we had shown that vitamin C supplementation effected an increase in serum immunoglobulin levels in humans. This observation permits two interpretations regarding a role for vitamin C in immunoglobulin synthesis. It may act as a primary, but nonspecific, stimulus for increased B cell activity. Alternatively, ascorbate may enhance the production of specific antibodies as a secondary effect subsequent to antigen-induced stimulation of a restricted population of B cells. This latter alternative is the object of our current investigations.

In establishing a role for vitamin C in the humoral immune response due regard must be had to the type of antigenic challenge. It is important to distinguish between T-dependent and T-independent antigens. Viral infections may introduce a further complication where lymphoid tissue is parasitized [8]. In this present paper we wish to report our findings on the effect of vitamin C supplementation on the production of antibodies to sheep red blood cells (S. R. B. C.) – a T-dependent cellular antigen. The earlier inconclusive studies [6, 7] had focused on scorbutic guinea pigs. However, for the manifestation of maximal beneficial effects PAULING [9] advocates a vitamin C intake in excess of minimal requirements. In line with this thinking we have, in our study, compared the performance of experimental animals on a minimal vitamin C intake with that of animals on ascorbate supplementation.

Methods and Materials

Twenty-six random-bred guinea pigs (young males, body mass 400–600 g) were randomly divided into 2 groups of 13 members each. All animals were depleted of tissue stores of vitamin C by feeding a specially-prepared ascorbate-free pelletized diet (Premier Milling Co., South Africa) for 2 weeks. The subsequent dietary regimen, which was maintained throughout the experimental period, may be summarised as follows:

a) All animals were fed ascorbate-free laboratory animal pellets. In addition, all animals were given a daily ration of 9 g shredded cabbage per animal. The vitamin C content of this portion of vegetable amounts to approximately 6 mg [2], which is equivalent to the daily turnover rate of ascorbate in these animals [1].

b) The «test» group of animals was provided with supplementary vitamin C (Merck, Art. 500074) in their drinking water, at a concentration of 2 g/litre (pH adjusted to 4.5 with NaOH/KOH; prepared fresh daily). Consumption of this water was allowed *ad libitum*. Assuming an average daily water consumption of 80 ml per animal [12], supplementary vitamin C intake amounted to 160 mg per animal per day.

Antigen

Red blood cells from one and the same sheep were employed in all experimental work described in this study. Washing of red cells and preparation of saline suspensions of erythrocytes followed described procedure [3].

Inoculation

This was effected by intraperitoneal injection of a 10 % saline suspension of S. R. B. C. The primary injection dose (in proportion to individual body mass) amounted to 6.0 ml/kg body mass and was administered two weeks after commencement of the dietary regimen as described in a) and b) above. Surviving animals were given a «booster» injection, dosed at 3.0 ml/kg body mass, 49 days after the primary injection.

Bleeding and Treatment of Serum Samples

Blood samples were obtained from all animals at intervals indicated in Table I. Bleeding was by cardiac puncture. Sera were preserved with NaN_3 (1 mg/ml) and stored frozen until required for assay. Serum samples were assigned a random number code to eliminate investigator bias. Decodification was effected only after all assays were completed.

Assay for Antibodies against S. R. B. C.

Serum concentration of anti-S. R. B. C. antibodies was determined by a haemolytic radial immunodiffusion technique, essentially as described by HIRAMOTO *et al.* [4]. We adopted the following slightly modified procedure:

Twelve millilitres of a 1 % solution of agar (Behring Institute, Hoechst) in borate-buffered-saline (B. B. S.) were equilibrated with 0.4 ml DEAE-dextran (10 mg/ml) and 1.0 ml of a 10 % saline suspension of S. R. B. C. at 52° C. Nine millilitres of the resultant mixture were poured into a plastic petri dish (diameter: 9 cm). After solidification, 2.5 mm diameter wells were punched into the gel. Wells were filled to the brim with suitable saline dilutions ($^1/_2$–$^1/_{20}$) of sera [3]. Radial diffusion of antibody was allowed to proceed for 44 hours at 4° C. Plates were then overlaid with 4 ml of 1 : 10 dilution in modified barbital buffer [3] of reconstituted guinea pig complement (Miles, Code No. 64–283–1) and incubated at 37° C for 4 hours. After washing with saline the ring diameters of lytic zones were measured on a «TG Calibrating Viewer» (Kallestad Laboratories, Inc.).

Quantitation of haemolytic antibody concentration was by reference to a «standard reference pool» of experimental sera. Four dilutions ($^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$) of «standard pool» serum were incorporated in duplicate in each plate. Undiluted «standard pool» serum was arbitrarily assigned a value of 400 haemolysin units. A linear relationship between haemolytic ring area and the square root of haemolysin concentration obtained, in accord with HIRAMOTO *et al.* [4].

All sera were assayed in duplicate in separate plates. Dilutions were adjusted such that measured ring diameters fell within the range covered by the «standard» dilutions (4.5–10.0 mm). Duplicate assays were allowed a ± 6 % margin of variability.

Statistics

Numerical results obtained were subjected to statistical analysis using student's t-test for paired samples. P values $< 0{,}03$ (one tail test) were regarded as statistically significant.

Results

The antibody response to S. R. B. C. in individual guinea pigs is shown in Table I. Statistical evaluation of mean antibody titres for each group of animals and for each bleed is summarized in Table II. A statistically significant difference in the antibody production between control and test animals is evident during the early phase of the primary response. Up to the fourth bleed (day 18) the serum antibody levels in the test animals are significantly higher than those

[3] Serum samples were previously heat-treated at 55° C for 20 minutes in order to inactivate endogenous complement.

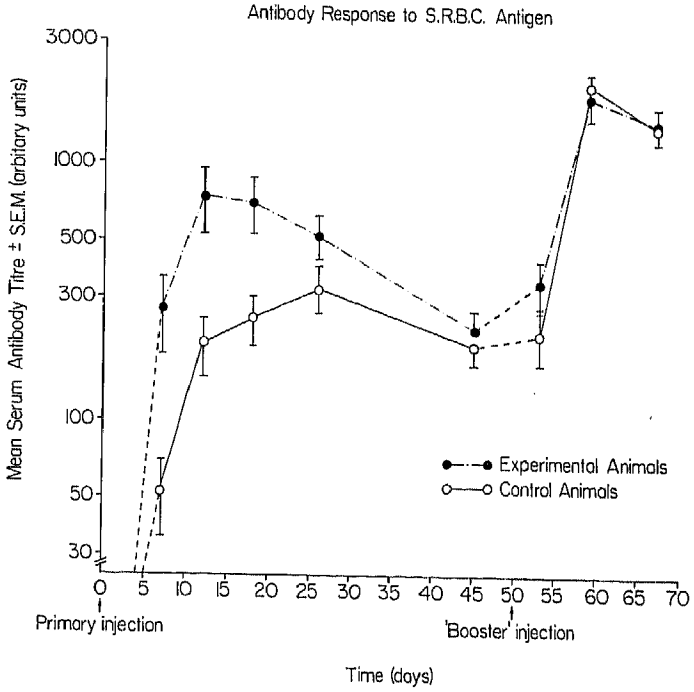
Tab. I: Serum Levels of Anti-SRBC Antibodies in Experimental Animals (Arbitrary Haemolysin Units – see Text)

	Bleed No.	I	II	III	IV	V	VI	VII	VIII	IX
	Days after primary injection	3	7	12	18	26	45	53	59	67
	Days after "booster" injection							4	10	18
	Guinea Pig No.									
Control Animals	1	N.M.*	140	300	412	390	172	215	2540	1932
	2	N.M.	N.M.	-	-	-	-	-	-	-
	3	N.M.	N.M.	N.M.	40	74	106	220	1347	737
	4	N.M.	88	185	-	-	-	-	-	-
	5	N.M.	50	175	246	242	104	82	1692	983
	6	N.M.	N.M.	97	101	102	82	127	2954	1820
	7	N.M.	-	-	-	-	-	-	-	-
	8	N.M.	80	180	296	435	195	-	-	-
	9	N.M.	N.M.	N.M.	104	247	229	-	-	-
	10	N.M.	112	639	-	-	-	-	-	-
	11	N.M.	N.M.	117	242	400	209	216	1640	1425
	12	N.M.	167	334	584	694	403	462	2058	1707
	13	N.M.	N.M.	170	216	349	280	-	-	-
Test Animals	14	N.M.	665	928	761	364	261	-	-	-
	15	N.M.	-	-	-	-	-	-	-	-
	16	N.M.	869	2359	2198	1061	255	558	1981	2012
	17	N.M.	53	206	325	313	115	-	-	-
	18	N.M.	112	129	194	175	95	155	1418	815
	19	N.M.	92	85	130	149	95	150	1986	1803
	20	N.M.	N.M.	223	324	408	-	-	-	-
	21	N.M.	112	217	283	365	190	215	1192	1053
	22	N.M.	N.M.	600	924	1144	212	-	-	-
	23	N.M.	223	1128	830	592	382	563	1227	1023
	24	N.M.	507	1031	857	537	247	430	3150	1950
	25	N.M.	349	1233	925	662	462	-	-	-
	26	-	-	-	-	-	-	-	-	-

* N. M.: not measurable (< 20 haemolysin units). A dash (–) indicates the animal has died.

Tab. II: Summary of Statistical Analysis on Serum Levels of Anti-S. R. B. C. Antibodies

	BLEED NO.	I	II	III	IV	V	VI	VII	VIII	IX
	Days after primary injection	3	7	12	18	26	45	53	59	67
	Days after booster injection	-	-	-	-	-	-	4	10	18
CONTROL ANIMALS	Mean Antibody titre (arbitrary Haemolysin units)	0	53	200	249	326	198	220	2037	1434
CONTROL ANIMALS	S.D.	0	62	179	169	189	101	131	609	482
CONTROL ANIMALS	S.E.M.	0	18	54	56	63	34	54	248	197
TEST ANIMALS	Mean Antibody titre (arbitrary Haemolysin units)	0	271	740	705	525	231	345	1826	1443
TEST ANIMALS	S.D.	0	292	691	585	327	120	196	739	535
TEST ANIMALS	S.E.M.	0	88	208	176	98	38	80	302	218
	P	-	<0.012	<0.012	<0.012	:0.06 (N.S.)	N.S.	N.S.	N.S.	N.S.



of the controls ($P < 0.01$). The peak response in the test animals occurred approximately 12 days after primary immunization. The corresponding time interval for peak response in the control animals was approximately 26 days. Furthermore, the peak antibody titre for the primary response in the test animals was significantly higher than that observed in the controls ($P < 0.03$).

In the latter phase of the primary immune response to S. R. B. C. the antibody titres of the test and control groups of animals became comparable. Similarly, a «booster» injection of S. R. B. C. did not evoke significantly different antibody responses. A comparison of the time-dependent appearance of anti-S. R. B. C. antibodies in the control and test animals is presented in Figure 1.

Discussion

The role of vitamin C in stimulating the antibody response to a T-dependent antigen (S. R. B. C.) appears to be restricted to the early phase of the primary humoral response. This strongly suggests an effect on IgM elaboration[cf] [10]. Immunoglobulin M is present in all vertebrates and is normally produced early in the immune response. However, its half-life (about 5 days) is significantly less than that of Ig G (about 20 dys) [13]. The latter class of antibody predominates in the later stages of the primary response as well as in the secondary response. If ascorbate influenced only Ig M elaboration this would explain our failure to observe any enhancement of antibody production in the later phases of the immune response. It must be admitted that our assay method for quantitating anti-erythrocyte antibodies, *viz*, haemolytic capacity, favours detection of Ig M. Be that as it may, Ig M is a most effective first line of defence against invading organisms precisely because of its superior lytic efficiency.

An incidental observation arising from our study-was the demise of three test-group animals within 2 hours of receiving the «booster» injection. The ostensible cause was anaphylactic shock, which is believed to be mediated by antigen-specific Ig E antibodies [5].

The enhancement of the immune response in guinea pigs described in this report may be attributed to a daily dose of 160 mg ascorbate per animal (see *Methods and Materials)* which is equivalent to 25 × the daily turnover rate in this species [1]. It is tempting, though not necessarily valid, to extrapolate our finding to humans in whom the ascorbate turnover rate is approximately 70 mg/day. On this basis, a comparable stimulation of the humoral immune response in humans could therefore be evoked with a daily vitamin C supplement of 1.5–2 g.

It is not possible to deduce from the present study whether ascorbate exerts a direct effect on B cells or whether its effect is mediated via helper T cells. A possible participation by T cells may be excluded by challenging the immune

system with T-independent antigen. This aspect is the object of a further study currently under way.

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