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Activation of Serum Complement Leads to Inhibition of Ascorbic Acid Transport (42530)

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Abstract. Ascorbic acid is transported into 3T6 fibroblasts by a carrier-mediated, energy-dependent saturable active process with a K_m of 112 μM and V_{max} of 158 pmole/min/mg protein. The transport is dependent on extracellular Na^+ concentration which reduces the K_m. It was recently observed in this laboratory that bovine serum contained a heat-labile factor which, after interaction with bacterial endotoxin (lipopolysaccharides), inhibited ascorbic acid transport (J. J. Alleo and H. Padh, Proc Soc Exp Biol Med **179**:128–131, 1985). We report here that the inhibition of ascorbic acid transport by endotoxin is mediated by the activation of serum complement. This was done by examining the activation of complement by other activators like zymosan and immunocomplexes (e.g., albumin and antibodies to albumin). Ascorbate transport was inhibited by the mixture of unheated serum and the activators. No inhibition was observed with serum devoid of C3 (component 3 of the complement). When C3-deficient serum was reconstituted by the addition of purified C3, the endotoxin-induced inhibition of ascorbate transport was restored. The implication of these findings is that in spite of a normal intake and blood level of the vitamin, tissues may not be getting adequate vitamin C during disease states when the complement in serum is activated. In other words, what may be considered an adequate intake of vitamin C under health conditions may not be adequate under disease conditions.

Ascorbic acid (vitamin C) is a dietary requirement for humans, primates, guinea pigs, and a few species of birds. One of the best known functions of ascorbic acid is as a cofactor in the hydroxylation reactions of lysine and proline in nascent collagen (1–4). Ascorbic acid is transported into collagen producing 3T6 fibroblasts, by an energy-dependent saturable active process with a K_m of 112 μM. The transport is dependent on extracellular Na^+ and it is suggested to be a Na^+-cotransport system (5). While studying ascorbate transport by 3T6 fibroblasts, it was observed that bovine serum contained a heat-labile factor which interacted with bacterial endotoxin and inhibited ascorbic acid transport (6). These data suggested the possibility that endotoxin activated the complement system present in unheated serum and the activation of serum complement generated factor which was inhibitory for ascorbate transport (6). The basis for this suggestion is the known ability of endotoxin to activate complement pathways and the similar heat lability (30 min at 56°C) of complement and the inhibitory factor.

Complement comprises a group of serum proteins which play a very critical role in eliminating foreign particles from blood, particularly infectious agents. The complement system can be activated by two distinct mechanisms: classical and alternate pathways (7–12). Some of the complement components are common to both pathways while some are specifically involved in only one of the two pathways. The process of activation involves a specific sequence of controlled proteolytic cleavage followed by assembly of the specific components into an organized group which attaches to foreign particles and facilitates their phagocytosis by leukocytes. There are a number of agents which are known to activate the complement cascades. Perhaps the most important among them which activates the classical pathway of complement is immunocomplex of antigen and its respective antibodies (in human, IgG1, 2, and 3 and IgM subtype). Among the agents which activate the alternate pathway are polysaccharides like zymosan, inulin, and glycogen and the bacterial endotoxins which are lipopolysaccharides in nature

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(7–12). In this paper we test the hypothesis that activation of serum complement generates factor that is inhibitory for ascorbic acid transport.

Materials and Methods. *Materials.* Antigens, antisera, and guinea pig serum devoid of C3 were obtained from Cooper Biomedicals, Malvern, Pennsylvania. Guinea pig C3 was purchased from Diamedix Corp., Miami, Florida. L-[1-^{14}C]Ascorbic acid was purchased from the Amersham Corp. Dulbecco–Vogt modification of Eagle's medium was obtained from GIBCO Laboratory. Zymosan was from Sigma and guinea pig serum was obtained from Miles Laboratory. Bovine serum was from GIBCO Laboratory and rabbit and mouse sera were purchased from Cappel Laboratory.

Cells. 3T6 fibroblast cells (ATCC No. CCL 96) were obtained from American Type Culture Collection, Rockville, Maryland. The cells were grown as described earlier (6).

Preparation of immunocomplexes. Antiserum was heat inactivated at 56°C for 30 min before being mixed with a respective antigen. Usually 10-μl aliquots of antigen solution (10 mg/ml) were added to 2 ml of ice-cold, heat-inactivated antiserum until it turned cloudy. It took between 50 and 100 μl of antigen solution to make visible immunocomplexes in 2 ml of antiserum. After 15 min on ice, the immunocomplexes were immediately used for the experiments shown in Table II.

Ascorbic acid transport. Cells were grown in 25-cm^2 tissue culture flasks. When the cells reached near confluency, the flasks were used for experiments. Monolayers were rinsed four times with 5 ml of balanced salt solution (BSS, containing 150 m*M* NaCl, 4.2 m*M* KCl, 1.0 m*M* NaH_2PO_4, 11.2 m*M* D-glucose, 0.7 m*M* $MgCl_2$, 2.0 m*M* $CaCl_2$, and 10 m*M* Hepes, adjusted to pH 7.2 with NaOH. To prevent oxidation of ascorbic acid 1 m*M* thiourea was added to BSS (13)). In the presence of 1 m*M* thiourea, there was no detectable degradation of ascorbic acid, when the ascorbate concentration was 2, 30, or 200 μ*M* in BSS. Under these conditions, bovine or guinea pig serum at 20% concentration, either unheated or heat inactivated, had no effect on the ascorbate stability ((13), and unpublished data).

The ascorbate transport was studied in a total volume of 2.5 ml. Unless indicated otherwise the cells were incubated for 15 min with various additions, as shown in the tables, before the transport was initiated by addition of L-[1-^{14}C]ascorbic acid. The concentration of radiolabeled ascorbate was 2.75 μ*M*. Unless indicated otherwise, the transport was terminated after 20 min of incubation at 37°C by removing the radioactive BSS and washing the cell sheet four times with 5 ml of ice-cold BSS. The washed cell sheet was dissolved overnight in 2.5 ml of 0.4 *N* NaOH. Two milliliters of the dissolved cells was used for counting radioactivity and an aliquot was used for protein determination by the method of Bradford (14). Other details of the transport were published earlier (6).

Results and Discussion. Ascorbic acid is transported by 3T6 fibroblasts by a carrier-mediated saturable active Na^+-cotransport process (5). Previous studies in this laboratory had shown that fetal bovine serum contained a heat-labile factor which, upon activation by endotoxin, inhibited the ascorbate transport by 3T6 fibroblasts (6). We therefore examined whether other sera contained the inhibitory factor. The data as shown in Table I suggested that sera from rabbit, mouse, and guinea pig also inhibited the ascorbate transport upon activation by endotoxin. However, the extent of inhibition differed widely from species to species. Guinea pig serum in the presence of

TABLE I. INHIBITION OF ASCORBATE TRANSPORT BY ENDOTOXIN: EFFECT OF SERA FROM DIFFERENT SPECIES

	Ascorbate transport percentage control
1. Bovine serum (GIBCO)	100
2. No. 1 + endotoxin	64
3. Guinea pig serum (Miles)	100
4. No. 3 + endotoxin	37
5. Rabbit serum (Cappel)	100
6. No. 5 + endotoxin	69
7. Mouse serum (Cappel)	100
8. No. 7 + endotoxin	78

Note. Cells grown to near confluency were incubated for 4 hr in a fresh medium containing 20% of the indicated serum ± endotoxin (200 μg/ml). The ascorbic acid transport was then studied by addition of radioactive ascorbate and followed by incubation for 1 hr at 37°C. Other experimental details have been published earlier (6). The ascorbate transport in the controls was 6.00 ± 0.25 pmole/min/mg protein.

endotoxin showed the strongest inhibition and was used for the subsequent studies. The data suggest that an inhibitory factor of ascorbate transport is present in various animal sera in an "inactive" form which is converted into an "active" form by endotoxin.

Since ascorbic acid and dehydroascorbic acid are interconvertible forms of vitamin C which are transported into cells by two different mechanisms, the question arises regarding the role of dehydroascorbic acid transport in the present studies. We believe, for the following reasons, that dehydroascorbic acid is not the transported species in the present study. Thiourea (1 m*M*) completely prevented the conversion of ascorbic acid to dehydroascorbic acid for up to 5 hr (13). Inclusion of 1 m*M* thiourea had no effect on the endotoxin-induced inhibition of ascorbate transport (unpublished data) suggesting that we are indeed studying only ascorbate transport. Furthermore, dehydroascorbic acid is believed to share a transport system with glucose (15). Under the conditions in which endotoxin inhibited ascorbic acid transport, it had no effect on the glucose transport (unpublished data). Similarly, insulin had no effect on the ascorbic acid transport under the same experimental conditions, suggesting that there was no detectable formation and subsequent transport of dehydroascorbic acid. Finally, the radioactivity extracted from the cells in metaphosphoric acid comigrated with the standard ascorbic acid, again suggesting that ascorbic acid is the transported species (6).

We tested the specificity of the effect of endotoxin on the ascorbic acid transport. It was conceivable that endotoxin in the presence of unheated serum causes some general membrane damage affecting the ascorbic acid transport in a nonspecific way. However, the cells in our study remained firmly attached to the flask surface during the experiments. There was no increase in cell floating and the cell viability as judged by trypan blue exclusion remained unaffected by the endotoxin treatment. In addition, the glucose transport remained unaffected under the conditions where ascorbate transport was inhibited by endotoxin. These results suggest that the inhibitory effect of endotoxin on the ascorbate transport is specific to ascorbate transport and not due to generalized membrane damage.

We then examined whether activation of serum complement by immunocomplexes led to inhibition of ascorbate transport. As shown in Table II, immunocomplexes in the presence of unheated guinea pig serum inhibited ascorbate transport by 3T6 fibroblasts. Immunocomplexes in the absence of serum or in the presence of serum inactivated at 56°C were ineffective. The ascorbate transport was linear under these conditions. Zymosan from yeast (16) at 5 and 10 mg/ml also caused 45 and 65% inhibition, respectively.

To ascertain the role of the activation of complement in the observed inhibition of ascorbate transport, the effect of endotoxin in the guinea pig serum devoid of component C3 of complement was examined. As shown in Table III, guinea pig serum devoid of C3 did not show any inhibition of ascorbate transport by endotoxin, unless reconstituted by purified C3, confirming the requirement of complement activation for inhibition of ascorbate transport.

These results strongly support that activation of complement inhibits ascorbic acid transport. However, it is also conceivable that activated complement may stimulate ascorbate secretion or efflux from cells thereby resulting in reduced accumulation of radioactive ascorbate. This appears unlikely because of the following reasons. The ascorbate transport was linear under these experimental conditions, arguing for the direct effect of activated complement on the ascorbate transport. To examine the alternate possibility of ascorbate secretion, the cells were incubated in BSS with radioactive ascorbate for 30 min, washed, and further incubated with or without guinea pig serum (20%) and endotoxin (200 μg/ml). Release of radioactivity from the cells into the medium was monitored for 30 min. Endotoxin in the presence or absence of guinea pig serum had no effect on the release of radioactivity accumulated by cells, ruling out any effect of activated complement on ascorbate secretion or efflux.

It is not known which component of the complement interacts with and inhibits ascorbate transport. From our results it is suggested that the inhibitor of ascorbate transport is generated during the activation of complement through the classical pathway (by immunocomplexes) as well as through the alter-

TABLE II. EFFECT OF IMMUNOCOMPLEXES ON THE ASCORBATE TRANSPORT BY 3T6 FIBROBLASTS

	Ascorbate transport (pmole/min/mg protein)	Percentage control
1. BSS	5.82 ± 0.21	100
2. BSS + guinea pig serum (0.5 ml)	5.65 ± 0.22	97
2 + endotoxin (200 μg/ml)	1.63 ± 0.10	28
2 + Immunocomplex A (50 μl)	4.95 ± 0.20	85
2 + Immunocomplex A (100 μl)	3.75 ± 0.11	65
2 + Immunocomplex A (250 μl)	1.16 ± 0.12	20
2 + Immunocomplex B (50 μl)	5.41 ± 0.20	93
2 + Immunocomplex B (100 μl)	4.37 ± 0.14	75
2 + Immunocomplex B (250 μl)	1.63 ± 0.10	28
2 + Immunocomplex C (100 μl)	3.85 ± 0.20	66
2 + Immunocomplex C (250 μl)	1.35 ± 0.14	23
3. BSS + immunocomplex A, B, or C (250 μl)	5.63 ± 0.16	97
4. 3 + heat-inactivated guinea pig serum (0.5 ml)	5.73 ± 0.17	98

Note. The values are mean ± standard deviation of four determinations. Immunocomplex A was human albumin and goat antiserum against human albumin. Immunocomplex B was human IgG and goat antiserum against human IgG. Immunocomplex C was guinea pig IgG and rabbit antiserum against guinea pig IgG. The cells were incubated with the respective additions for 15 min at 37°C before the transport of ascorbate was initiated by addition of radioactive ascorbic acid. Other details are given under Materials and Methods.

nate pathway (by endotoxin, zymosan). This would imply that the factor is generated at or beyond the C3 step of complement activation; the point which is also supported by the results obtained with C3-deficient serum. The details of the mechanism of inhibition of ascorbate transport by the inhibitory factor remains unknown. The inhibitor may either inhibit the transport directly by interacting with the ascorbate transporter or may inhibit the transport indirectly by altering one or more of the conditions that affect ascorbate transport (5), for example, the electrochemical gradient of Na^+ across the plasma membrane. Our results, as discussed earlier, indicated that there does not seem to be general damage to the membrane and the inhibitory effect seems to be specific to the ascorbate transport.

It is interesting to realize that the activation of complement leads to inhibition of ascorbate transport, which could lower tissue supply and the level of vitamin C. The metabolic consequences of lowered ascorbic acid supply to the tissues are not known. However, it opens up the question of the adequacy of ascorbic acid supply to tissues under conditions like those of infections and autoimmune diseases when the serum complement is activated.

There are several implications of these findings. First, they demonstrate the presence of

TABLE III. EFFECT OF C3-DEFICIENT SERUM ON THE ENDOTOXIN-INDUCED INHIBITION OF ASCORBATE TRANSPORT

	Ascorbate transport (pmole/min/mg protein)	Percentage control
1. BSS	6.12 ± 0.15	100
2. BSS + guinea pig serum − C3 (0.5 ml)	5.75 ± 0.14	94
3. No. 2 + endotoxin (200 μg/ml)	5.88 ± 0.15	96
4. No. 2 + C3 (0.2 mg)	5.60 ± 0.13	92
5. No. 4 + endotoxin (200 μg/ml)	2.70 ± 0.12	44
6. Like No. 4 except that heat-inactivated guinea pig serum − C3 was used.	5.92 ± 0.17	97

Note. The cells were incubated with respective additions for 15 min at 37°C before the ascorbate transport was initiated by addition of radioactive ascorbic acid. Other details are given under Materials and Methods.

serum complement factor, which inhibits ascorbic acid transport when activated. To the best of our knowledge, no factor in serum has ever been demonstrated which affects the transport of ascorbic acid to the tissues. In an earlier study (17), it was shown that ascorbic acid uptake by leukocytes, *in vitro,* markedly varied in plasma from different individuals. No further characterization of this effect has been studied.

The characteristics of ascorbate transport are remarkably similar in all the cell types studied. Like fibroblasts, other cells exhibit an energy-dependent and Na^+-dependent active transport of ascorbic acid (18–22). If the inhibitory factor affects ascorbate transport in other cells as well, the supply of ascorbic acid to these tissues could be jeopardized during autoimmune conditions and infections when the serum complement is activated. Among all nutrients, the determination of adequate nutritional requirements for vitamin C has sparked the most intense discussion. While the officially recommended daily allowance for adults in the U.S.A. is 60 mg, a quantity of up to several grams is recommended by some experts in the professional as well as the public press. Our results suggest that what may be considered adequate intake of vitamin C in a healthy state may not be enough during infections because of the disrupted supply of ascorbic acid to the tissues. This could be the case even when vitamin C intake and blood level are normal.

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