ASCORBIC ACID AND THE IMMUNE RESPONSE

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INTRODUCTION

Since the introduction of ascorbic acid as an antiviral and antibacterial agent (Klenner, 1951; McCormick, 1952; Klenner, 1974) interest has focused on the possible immunologic mechanisms involved in its protective effect. The role of ascorbic acid in the immune response is reviewed here with regard to cellular and humoral functions, and experiments pertaining to the role of ascorbic acid in autoimmunity and anaphylaxis are discussed.

The structure of ascorbic acid is shown in Figure 1. The reduced form (ascorbic acid) is a hexose sugar with an ene-diol moiety; loss of one electron results in oxidation to the ascorbate free radical, while loss of two electrons results in the formation of dehydroascorbic acid. In both processes, the released electrons may be utilized to quench various free radicals. Thus, during the oxidation of ascorbic acid to dehydroascorbic acid, two molecules of free radicals may be quenched, which underscores the importance of ascorbic acid with regard to its antioxidant properties. In fact, ascorbic acid is only slightly less effective in quenching superoxide anion radical than is the enzyme superoxide dismutase, when considered at physiological concentrations (Leibovitz and Siegel, 1980). Further, both the ascorbate free radical and dehydroascorbic acid may be reduced to ascorbic acid by the enzymes NADH:semidehydroascorbate reductase and dehydroascorbate reductase, respectively (Figure 1), which provides an effective recycling mechanism. We have recently reviewed the role of ascorbic acid as a free radical quencher in biological systems (Leibovitz and Siegel, 1980).

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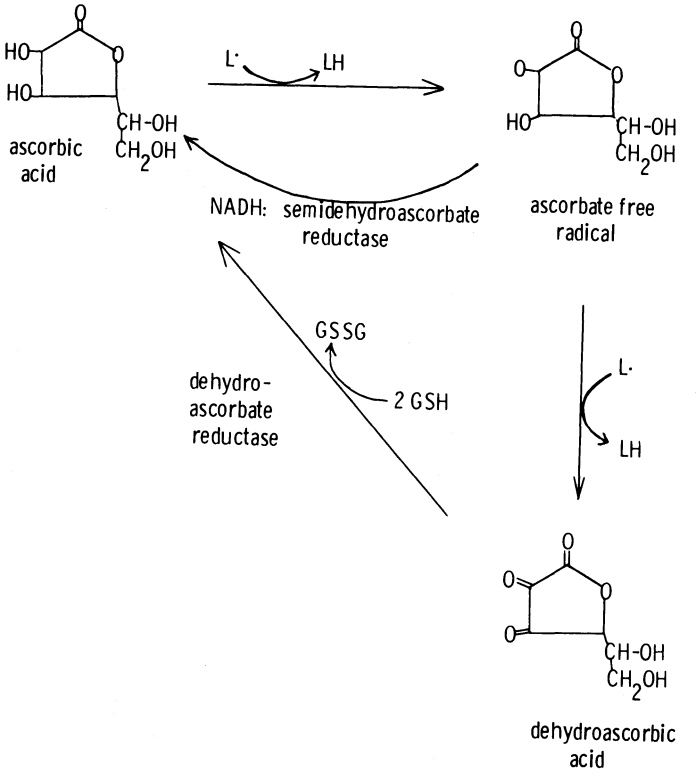

FIGURE 1. Structure of Ascorbic Acid and Its Oxidation Products.

ASCORBIC ACID AND T-LYMPHOCYTE FUNCTION

The activity of T-lymphocytes is well known to be affected by ascorbic acid. Mueller et al. (1962) noted that vitamin C deprivation afforded protection against the induction of experimental allergic encephalomyelitis, a T-cell-mediated disease. Moreover, the degree of protection was directly proportional to the duration of vitamin C deficiency. In the scorbutic guinea pig a marked suppression of the tuberculin reaction has also been observed (Mueller and Kies, 1962; Zweiman et al., 1966). Interestingly, the anergy of the scorbutic guinea pig to tuberculin reactions may be at the efferent level: passive transfer of peritoneal exudate cells from scorbutic guinea pigs was noted to transfer tuberculin sensitivity to normal recipients, while transfer of cells from tuberculin-sensitive donors to scorbutic guinea pigs did not induce tuberculin sensitivity (Zweiman et al., 1966). These results may indicate anergy to tuberculin reactions at the level of the inflammatory response, as is discussed in detail below.

We have examined the effect of supplementary ascorbic acid on mitogen-induced T-lymphocyte blast transformation in the mouse, and have noted a significant enhancement in Con A-induced blast transformation of splenic lymphoid cells in BALB/c mice given high doses of dietary ascorbic acid (250 mg% in the drinking water) (Siegel and Morton, 1977). Mice, unlike humans and guinea pigs, produce their own ascorbic acid, and our results described above therefore indicate a pharmacological effect rather than a physiological effect of ascorbic acid. These results have been confirmed in various laboratories. Thurman and Goldstein (1979) have reported a marked depression in T-lymphocyte blast transforamtion in guinea pigs given a scorbutic diet, while Yonemoto et al. (1976) observed a significant enhancement in PHA-induced T-lymphocyte blast transformation in humans given 5 g ascorbic acid per day for 3 days. More recently, Anderson et al. (1980) have reported a significant enhancement of PHA- and Con A-induced blast transformation in lymphocytes from humans given 1-3 g of ascorbic acid per day. *In vitro*, however, ascorbic acid has been noted to cause a dose-dependent inhibition of PHA-induced blast transformation in human lymphocytes (Ramirez et al., 1979), suggesting an *in vitro* cytotoxic effect of ascorbic acid. Vitamin C deficiency has also been noted to diminish other T-lymphocyte-mediated activities, including allograft survival (Kalden and Guthy, 1972) and cell-mediated cytotoxicity (Anthony et al., 1979).

ASCORBIC ACID AND ANTIBODY PRODUCTION

Ascorbic acid may play a role in antibody production; however, conflicting reports make analysis of this phenomenon difficult. With regard to the plaque-forming cell (PFC) capacity of the spleen, we have observed that supplementary ascorbic acid (250 mg% in the drinking water) did not affect the PFC response to LPS and sheep erythrocytes (SRBC) in BALB/c mice (Siegel and Morton, 1977). Thurman and Goldstein (1979), however, have noted a diminished PFC response to SRBC in scorbutic guinea pigs, an effect which could be reversed by vitamin C supplementation. Long (1950) observed that the primary antibody response of guinea pigs to alum-precipitated diphtheria toxoid was unaffected by vitamin C deficiency; however, secondary responses were reduced in the deficient animals. Kumar and Axelrod (1969) have repeated the study by Long, and have reported no detrimental effect of severe vitamin C deficiency on either primary or secondary antibody responses to diphtheria toxoid. More recently, Anthony et al. (1979), using guinea pigs immunized with chicken erythrocytes, observed no difference in antibody titers among vitamin C-deficient, pair-fed, or ad libitum-fed controls.

In contrast, supplementation of normal humans with 1 g/day ascorbic acid has been noted to increase serum levels on non-specific IgG and IgM (Prinz et al., 1977; Vallance, 1977).

Further, significant positive correlations were observed between leukocyte ascorbic acid levels and IgG and IgM in humans (Vallance, 1977). Because of the disparity in results, no conclusions can be drawn concerning the action of ascorbic acid in antibody formation.

ASCORBIC ACID AND PHAGOCYTE FUNCTION

Recent studies have demonstrated the importance of ascorbic acid with regard to the activity of phagocytic cells. It has long been known that polymorphonuclear leukocytes (PMNL's) contain very high levels of vitamin C (Crandon et al., 1940). Further, the spleen, a highly phagocytic organ, has been noted to contain 30-35 mg vitamin C per 100 g wet weight, which was higher than the levels reported for most organs (Zannoni et al., 1974). We have also observed high levels of vitamin C in the spleen of mice which increased with advancing age (Siegel and Leibovitz, 1979). In DBA/a females, for example, 1 month levels were observed to be 28 mg per 100 g, 14 month levels 41 mg per 100 g, and 27 month levels were 50 mg per 100 g. This may be contrasted to a decline in serum and liver vitamin C levels with advancing age in the mouse (Siegel and Leibovitz, 1979), which suggests an important role for vitamin C in splenic function. In this regard, significant negative relationships were observed between spleen vitamin C levels and spleen weight, as discussed below. DeChatelet et al. (1974) have observed that PMNL's and macrophages from rabbits or guinea pigs concentrated vitamin C by a factor of 10-40 over serum. They also noted that normal and BCG-induced rabbit alveolar macrophages contain approximately 5 fold more vitamin C per 10^8 cells than did rabbit peritoneal PMNL's, indicating a greater requirement of macrophages for ascorbic acid.

The chemotactic response of animal and human PMNL's as well as macrophages is clearly related to vitamin C status. The *in vitro* enhancement of chemotaxis by ascorbic acid has been observed in PMNL's (Goetzl et al., 1974; Anderson and Theron, 1979) and in monocytes and eosinophils (Goetzl et al., 1974). Interestingly, Goetzl et al. (1974) also observed an enhancement of phagocyte chemotaxis by glutathione, which suggests that reducing agents may be effective with respect to chemotaxis. In normal humans, supplementation with 2-3 g/day of ascorbic acid was noted to enhance PMNL chemotaxis (Anderson et al., 1980). Macrophages isolated from vitamin C-deficient guinea pigs were observed to show a reduced migration on a glass surface when compared to macrophages from normal guinea pigs (Ganguly et al., 1976).

Ascorbic acid may also be an important chemotactic stimulant in certain phagocytic diseases. Boxer et al. (1976) have reported a stimulatory effect of supplementary ascorbic acid (200 mg/day) on PMNL chemotaxis in a patient with the Chediak-Higashi syndrome

(CHS), a disease characterized by recurrent infections. More recently, Gallin et al. (1979) examined the effect of supplementary ascorbic acid (600 mg/kg body weight) on PMNL function in mice with the CHS ("beige" mutant of C57Bl/6N), and have reported improved PMNL chemotaxis and bactericidal activity following such treatment. In two humans with the CHS, however, a lower dose of ascorbic acid (85mg/kg body weight) was without effect with respect to chemotaxis and bactericidal activity (Gallin et al., 1979). It has been suggested (Boxer et al., 1976) that the enhancing effect of ascorbic acid on chemotaxis in the CHS may be due, in part, to a decrease in the levels of cAMP, which in turn regulate microtubule formation and degranulation.

Phagocytosis of microbes may be related to ascorbic acid status: however, further studies are required before definitive statements are justified. Cottingham and Mills (1943) observed that peritoneal PMNL's from scorbutic guinea pigs showed significantly reduced ingestion of type I pneumococcus *in vivo*. The phagocytosis of a beta-hemolytic streptococcus *in vitro* was also observed to be dependent on the dietary ascorbic acid status (Nungester and Ames, 1948). Chatterjee et al. (1975a) noted a decreased phagocytosis of *Bacillus subtilis in vitro* in PMNL's isolated from scorbutic guinea pigs; supplementation with ascorbic acid (5 mg/100 g body weight/day) reversed this effect. Interestingly, large doses of ascorbic acid (300 mg/100 g body weight/day) resulted in only a modest increase in phagocytosis compared to PMNL's from scorbutic guinea pigs, suggesting a possible toxic effect of massive doses of ascorbic acid.

In contrast, Ganguly et al. (1976) have examined the effect of a vitamin C-deficient diet on functional characteristics of peritoneal macrophages, and have reported no significant impairment in the *in vitro* phagocytosis of *Staphylococcus aureus* in macrophages isolated from the deficient guinea pigs. More recently, Anderson (1979) has investigated the effect of ascorbic acid and its salts on the *in vitro* phagocytosis of *Candida albicans* by human PMNL's. Phagocytosis was unaffected by ascorbic acid concentrations of 10^{-6} to 10^{-2} M, whereas at higher levels an inhibitory effect was observed. In light of the conflicting reports described above, further study appears warranted with respect to the possible role of ascorbic acid as a modulator of phagocytosis.

The antimicrobial activity of phagocytes depends primarily on oxygen-dependent mechanisms, specifically the myeloperoxidase (MPO) system and the system for the generation of oxygen free radicals. The latter system employs electrons from NADPH for the univalent reduction of oxygen to superoxide radical (SOR), and is under the influence of the enzyme NADPH oxidase. Subsequent reactions lead to the production of hydrogen peroxide, hydroxyl radicals, and singlet oxygen, all of which possess antimicrobial activity

(Rosen and Klebanoff, 1979). The enzymatic and non-enzymatic processes leading to the formation of these products has been recently reviewed (Leibovitz and Siegel, 1980). The critical step in the production of oxygen free radicals is the production of NADPH, which is formed during hexose monophosphate (HMP) shunt activation. In this regard, it has been observed that the activity of the HMP shunt is, in part, dependent on the ascorbic acid status. The *in vitro* addition of ascorbic acid has been shown to enhance HMP shunt activity in PMNL's as well as macrophages as measured by the conversion of glucose-1-^{14}C to $^{14}CO_2$ and by the reduction of nitroblue tetrazolium (NBT), which depends on SOR formation (DeChatelet et al., 1971; 1972; Goetzl et al., 1974; Anderson, 1979). A possible mechanism of action has been suggested by DeChatelet et al. (1972), in which dehydroascorbic acid oxidizes glutathione (GSH) to the disulfide form (GSSG), which oxidizes NADPH to $NADP^+$ under the influence of the enzyme glutathione reductase, and the newly-formed $NADP^+$ stimulates HMP shunt activity. Oral supplements of ascorbic acid (0.2-2.0 g/day) have been reported to enhance HMP shunt activity in normal humans (Shilotri and Bhat, 1977); however, Anderson et al. (1980) have been unable to confirm these results using 1-3 g/day of ascorbic acid in normal humans. In humans treated with cortical steroids, ascorbic acid (2 g/day) was noted to elevate HMP shunt activity to normal, which suggests a possible role for supplementary ascorbic acid in immunocompromised patients (Chretien and Garagusi, 1973).

The MPO system, on the other hand, appears to be inhibited by ascorbic acid. McCall et al. (1971) noted a complete inhibition of iodination by the MPO system following the addition of 10 mM ascorbic acid *in vitro*. Smith et al. (1975) have confirmed these findings at concentrations of 5-20 mM; however, at lower levels (less than 5 mM), iodination was only partially inhibited. Since the levels of ascorbic acid found in leukocytes are below 5 mM, the physiological significance of these findings remains obscure. In this regard, Anderson et al. (1980) have observed no impairment from normal subjects receiving 1-3 g/day of ascorbic acid.

Despite an enhancement in the production of microbicidal oxygen radicals by ascorbic acid, most investigators have observed no relationship between microbicidal activity of phagocytes *in vitro* and ascorbic acid status *in vivo*. Shilotri and Bhat (1977), for example, observed no effect of ascorbic acid supplements (200 mg/day) on the *in vitro* killing of *E. coli* by human leukocytes; higher doses (2 g/day), however, were observed to inhibit bactericidal activity, an effect which was reversed following cessation of ascorbic acid supplementation. Stankova et al. (1975) have reported that scorbutic guinea pig PMNL's killed *Staphylococcus aureus in vitro* as well as control PMNL's, indicating that severe vitamin C deficiency does not impair microbicidal activity *in vitro*. The *in vitro* addition of 5 mM

ascorbic acid has been noted to be without effect on the killing of Staphylococcus aureus by human leukocytes; at 50 mM, however, an inhibitory effect was noted (Smith et al., 1975). McCall et al. (1971), however, noted no effect of 10 mM ascorbic acid on the in vitro killing of Staphylococcus aureus and E. coli by human leukocytes.

Ascorbic acid may, however, play a role in enhancing microbicidal activity in immunosuppressed hosts. In this regard, Olsen and Polk (1977) have reported that the in vitro addition of ascorbic acid partially reversed the inhibitory effect of hydrocortisone on the bactericidal activity of human PMNL's against Staphylococcus aureus, suggesting that cortical steroid-induced immunosuppression may be mitigated, in part, by exogenous ascorbic acid. In a patient with the CHS, Boxer et al. (1976) have reported that ascorbic acid (approximately 50 mg/kg body weight) raised the bactericidal activity of PMNL's against Staphylococcus aureus to normal levels. Gallin et al. (1979) were unable to confirm the enhancement of bactericidal activity in vitro by ascorbic acid supplements (approximately 85 mg/kg body weight) in two adults with the CHS; however, using a mouse model system, higher doses (600 mg/kg body weight) proved effective in enhancing bactericidal activity against Staphylococcus aureus in vitro and prolonging the the survival of mice infected with Candida albicans.

Thus, the bactericidal and fungicidal activity of PMNL's does not appear to relate to the ascorbic acid status, with the possible exception being immunosuppressed hosts. This lack of effect of ascorbic acid has led to the suggestion that ascorbic acid constitutes a functional part of the PMNL redox system and may function to protect cell constituents from denaturation by oxygen radicals produced during phagocytosis (Stankova et al., 1975). That PMNL's do produce various oxygen radicals, including SOR and hydroxyl radical, has been reported by numerous investigators, and has recently been reviewed by Tauber et al. (1979). Further PMNL's have been noted to show damage resulting from phagocytosis of bacteria, suggesting that oxidizing radicals produced during phagocytosis may impair the capacity of PMNL's for antimicrobial activity (Matheisz and Allen, 1979). This has led to the suggestion (Baehner et al., 1977) that autooxidation of PMNL's may result in altered functions, such as diminished bactericidal activity and impaired phagocytosis. The addition of various radical and peroxide scavengers in vitro and supplements of alpha-tocopherol (vitamin E) in normal humans has been noted to reduce autooxidative damage to PMNL's (Salin and McCord, 1975; Baehner et al., 1977). Thus, ascorbic acid, a well known free radical quencher (Leibovitz and Siegel, 1980), may function to protect phagocytes from oxidants produced during phagocytosis and oxidative killing. In this regard, ascorbic acid has been noted to be effective in reducing the secretion of SOR by guinea pig

macrophages (Oyanagui, 1976).

ASCORBIC ACID AND VIRAL INFECTIONS

Ascorbic acid appears to inhibit the growth of viruses both directly and indirectly. With regard to the common cold, numerous investigators have focused on the possible protective effect of ascorbic acid supplements, the results of which have been summarized by Pauling (1976). He reported a 36 percent reduction in integrated morbidity (amount of illness per person) for subjects receiving an average of 1 g ascorbic acid pe day, and suggested that higher doses might provide a greater protective effect. More recently, Baird et al. (1979), in a double-blind study of the effect of orange juice and placebo on morbidity associated with the common cold in 362 healthy adults, found a protective effect in the orange juice-treated group which was statistically significant. Interestingly, the orange juice provided only 80 mg ascorbic acid per day, suggesting that larger doses may have provided greater protection. These results taken together indicate a protective effect of ascorbic acid against the common cold; however, it is unlikely that ascorbic acid is beneficial in all circumstances, and it should not be viewed as a panacea for such infections.

Ascorbic acid has also been reported to be of benefit against other viruses. Murphy et al. (1974) examined the effect of dietary ascorbic acid in parainfluenza infection in the cotton-topped marmoset, a non-human primate, and observed a delay in the onset of disease, reduced clinical responses to infection, and a decreased mortality in the ascorbic acid-treated group. The dose of ascorbic acid was 0.5 g/kg body weight/day, which was observed to significantly increase blood vitamin C levels. Banic (1975) has investigated the effect of ascorbic acid supplements (200 mg/kg/day) on rabies virus infection in the guinea pig, and has noted a significant reduction in mortality in the ascorbic acid-treated group. In humans, an ascorbic acid-bioflavonoid complex (600 mg of each/day) has been reported to reduce vesiculation and to prevent the disruption of the vesicular membrane in herpes labialis infections when compared to lactose-treated controls (Terezhalmy et al., 1978). Higher doses, 1000 mg ascorbic acid and 1000 mg bioflavonoids, did not provide greater protection. Ascorbic acid supplements have also been reported to be of benefit in post-transfusion hepatitis, measles, mumps, herpes zoster, stomatitis aphthosa, and other viral infections in humans (Murats, 1975).

In vitro, ascorbic acid has been noted to inhibit the growth of rhinovirus in WI-38 fibroblasts (Schwerdt and Schwerdt, 1975), as well as increasing the resistance of chick embryo tracheal

cultures to infection by coronavirus (Atherton et al., 1978). Ascorbic acid has also been noted to inactivate a wide variety of bacterial viruses in vitro (Murata et al., 1971; 1972; 1975).

With regard to the mechanism of its possible antiviral activity, we have observed an increase in interferon production by ascorbic acid following stimulation of mouse L cell cultures with poly (rI)·poly (rC) (Siegel, 1975). We have also noted an enhancement of interferon production in vivo following stimulation with Rauscher leukemia virus in BALB/c mice treated with 250 mg% L-ascorbic acid in the drinking water (Siegel, 1974). Thus, the antiviral activity of ascorbic acid may be due, in part, to enhanced interferon production; however, the mechanism of this effect remains to be elucidated.

EFFECT OF PHAGOCYTOSIS ON TISSUE VITAMIN C LEVELS

Phagocytosis by PMNL's or macrophages is well known to result in the production of oxygen free radicals which may lead to auto-oxidation and diminished microbicidal activities, as discussed above. We have therefore investigated the effect of various doses of sheep erythrocytes (SRBC's) on serum and tissue vitamin C levels in order to ascertain the effect of acute phagocytosis on the levels of one free radical quencher, namely ascorbic acid. The results of these determinations are shown in Table I for DBA/2 x BALB/c F_1 female hybrids given three doses of SRBC's. Total vitamin C levels (ascorbic acid, dehydroascorbic acid, and 2,3-diketogulonic acid) were measured by the Lowry et al. (1945) modification of the method of Roe and Kuether (1943) in the spleen, thymus, liver, and serum four days following intraperitoneal injection of SRBC's. Serum and thymus vitamin C levels were unaffected by such treatment; however, spleen and liver vitamin C levels exhibited marked decreases. Dose-dependence was not, however, observed, and a dose of 10^7 SRBC's was as effective as 10^9 SRBC's with respect to lowering spleen and liver vitamin C values. Plaque-forming cell assays for antibody to SRBC's, which were also performed on these mice, did however evidence dose-dependence, with 17.8, 312, and 691 PFC/mg spleen for 10^7, 10^8, and 10^9 SRBC's, respectively. Since both the spleen and liver are phagocytic organs, these results suggest that phagocytosis may diminish vitamin C levels, a finding which is in accord with current knowledge concerning phagocytic events. Two free radicals, the hydroxyl radical and SOR, known to be produced during phagocyte activation, are also known to be quenched by ascorbic acid (Nishikimi, 1975; Fessenden and Verma, 1978). These results support the concept that ascorbic acid acts to quench oxygen free radicals in phagocytes, and may therefore function to retard auto-oxidative processes in these cell types.

TABLE I

Effect of Various Doses of Sheep Erythrocytes on Serum and Tissue Vitamin C Levels in Female DBA/a x BALB/c F_1 Hybrids[a]

Dose	Vitamin C (mg/100 grams wet weight) ± S.E.M.			
	Serum[b]	Liver	Thymus	Spleen
0	1.83±0.06	26.8±0.88	38.3±1.03	35.1±0.46
10^7	1.80±0.01[e]	17.6±0.65[d]	35.4±1.70[e]	30.0±1.01[c]
10^8	1.97±0.14[e]	18.6±0.45[d]	36.5±1.84[e]	28.1±0.65[d]
10^9	1.85±0.05[e]	17.8±0.80[d]	35.8±1.08[e]	27.7±0.92[d]

[a] DBA/2 x BALB/c F_1 female hybrids, two months of age, were injected i.p. with 0.20 ml saline or saline with the individual suspensions of washed sheep erythrocytes 4 days before sacrifice. Each group was composed of 4 mice, except the control group, which comprised 7 mice. Tissues were removed following exsanguination, homogenized in 10% trichloroacetic acid, and assayed for total vitamin C as described in the text within 7 days of preparation.

[b] Values shown represent mg vitamin C per 100 ml serum.

[c] Significantly different from controls with $p < 0.005$.

[d] Significantly different from controls with $p < 0.001$.

[e] Not significantly different from controls ($p > 0.05$).

VITAMIN C AND AUTOIMMUNITY

Previous studies in our laboratory have utilized the New Zealand Black (NZB) mouse strain as a model of autoimmune disease (Siegel and Morton, 1970; Morton and Siegel, 1980). We now report results of experiments which suggest a possible role for vitamin C in autoimmunity in the NZB mouse. Old NZB mice exhibit lower spleen vitamin C levels than do non-autoimmune strains (DBA/a and BALB/c), as is shown in Table II. In each instance, NZB mice showed significantly lower levels of vitamin C in the spleen when compared to non-autoimmune mice, suggesting a possible relationship between depressed spleen vitamin C levels and autoimmunity. However, it is likely that chronic phagocytosis of autoimmune disease, accounts for the observed reduction in spleen vitamin C levels in the NZB strain. This concept is in accord with the data presented in Table I, in which immunization with SRBC's resulted in lower vitamin C levels in the spleen and liver, two highly phagocytic organs.

TABLE II

Comparison of Spleen Vitamin C Levels
Between Old NZB, DBA/2, and BALB/c Mice

Group	Age (mo.)	Number of mice	Spleen Vitamin C[a]	P value
NZB females	20	7	30.5±2.92	<0.025
DBA/2 females	21	5	40.6±1.25	
NZB females	20	7	30.5±2.92	<0.005
BALB/c females	21	8	43.5±2.36	
NZB males	20	6	32.6±1.22	<0.005
DBA/2 males	27	4	44.4±2.44	

[a] Values expressed as mg per 100 grams wet weight of spleen ± standard error of the mean.

Spleen weight, an index of autoimmune progression, is inversely related to spleen vitamin C levels, as is illustrated in Figure 2 in the case of old, autoimmune NZB males and females. The relationship between spleen weight and spleen vitamin C was highly significant, with a correlation coefficient of -0.7636 ($p \ll 0.001$). This inverse relationship is not unique to NZB mice; we have observed significant negative correlations between spleen weight and spleen vitamin C levels in a variety of situations. In 4 month-old untreated DBA/a females, for example, a significant ($p<0.01$) inverse relationship was noted between spleen weight and spleen vitamin C levels, as illustrated in Figure 3. Similar relationships were observed in 4 month-old DBA/2 males treated with 30% deuterium oxide ($r= -0.859$, $p<0.01$), in 15 month-old DBA/2 females infected with 0.5% Rauscher leukemia virus ($r= -0.769$, $p<0.005$), and in 4 month-old BALB/c males infected with 10%, 1%, and 0.1% Rauscher leukemia virus ($r= -0.836$, $p<0.001$). Thus, the inverse relationship between spleen weight and spleen Vitamin C levels appears to be a general phenomenon in the mouse. Other investigators have reported similar findings in the guinea pig and in humans. Bates et al. (1978) have noted that vitamin C-deficient guinea pigs exhibited a 5 fold increase in spleen weight compared to pair-fed and ad libitum-fed controls. This splenomegaly was associated with a marked decrease in spleen vitamin C levels in the vitamin C-deficient group. In humans, MacLennan and Hamilton (1976) have noted a significant inverse

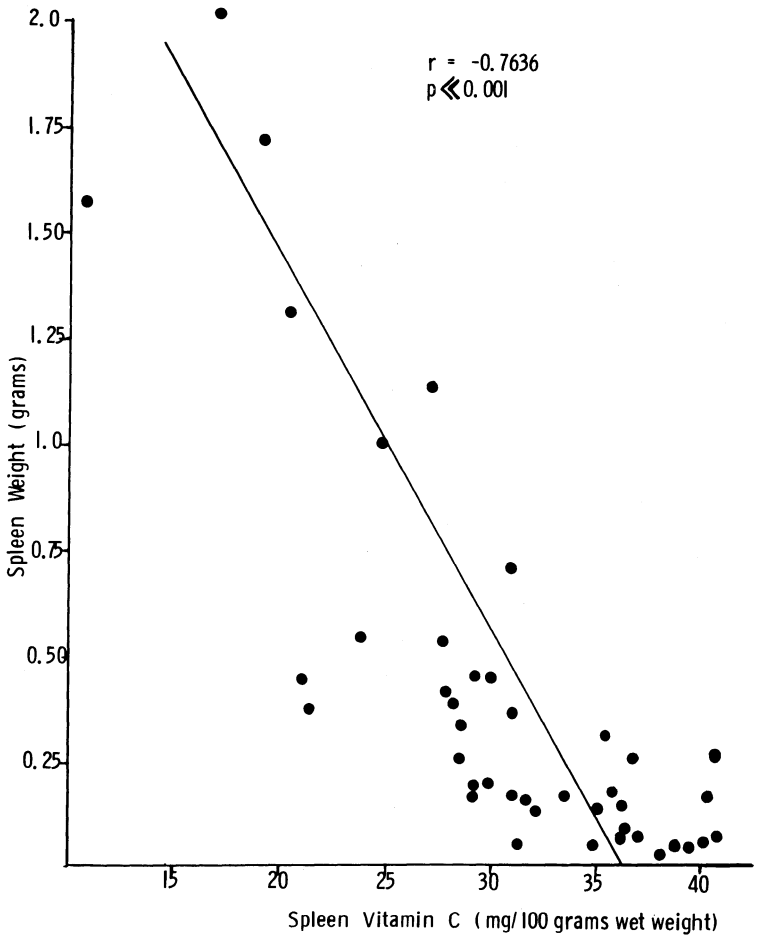

FIGURE 2. Linear Regression Analysis of Spleen Weight as a Function of Spleen Vitamin C in Old, Autoimmune NZB mice.

relationship between white blood cell counts and leukocyte counts and leukocyte vitamin C levels. Further, normal humans who supplement their diet with 2-3 g per day of ascorbic acid have been noted to evince lower leukocyte counts than those who do not (Robinson et al., 1975). These observations in the mouse, guinea pig, and human suggest that higher vitamin C levels in splenic and peripheral leukocytes reduces the requirement for such cell types, and may underscore the importance of vitamin C in leukocyte function.

We have also examined the relationship between spleen weight and the ratio of oxidized to reduced (ox/red) vitamin C in the

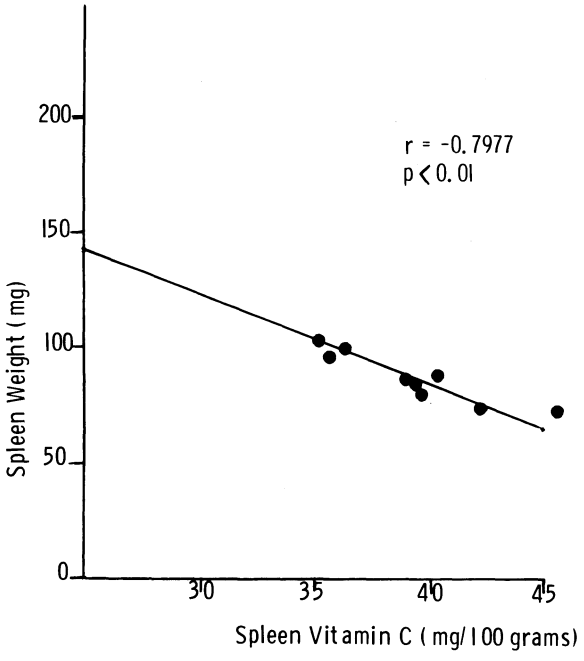

FIGURE 3. Linear Regression Analysis of Spleen Weight as a Function of Spleen Vitamin C in Four Month-Old, Untreated DBA/2 Female Mice.

spleen of NZB mice (Figure 4). A significant positive association was observed between these parameters, indicating that the metabolism of ascorbic acid to the oxidized form (dehydroascorbic acid and 2,3-diketogulonic acid) is increased during spleen enlargement in the NZB strain. We have previously observed that the ox/red vitamin C ratio reflects oxidative damage following carbon tetrachloride administration and during in vivo aging, which has led to the suggestion that the ox/red vitamin C ratio may be a useful measure of in vivo aging (Leibovitz, 1979). In the case of NZB autoimmune disease, the elevated spleen ox/red vitamin C ratios in mice with splenomegaly may reflect phagocytosis-induced free radical production which is known to oxidize ascorbic acid. These results are consistant with both the lowering of total vitamin C levels during splenomegaly in the NZB and with the reduction of total vitamin C levels subsequent to immunization with SRBC's.

NZB mice occasionally survive to very old ages, and we have compared parameters of disease and total vitamin C levels of 24-26 month-old NZB females with 16-20 month old autoimmune NZB

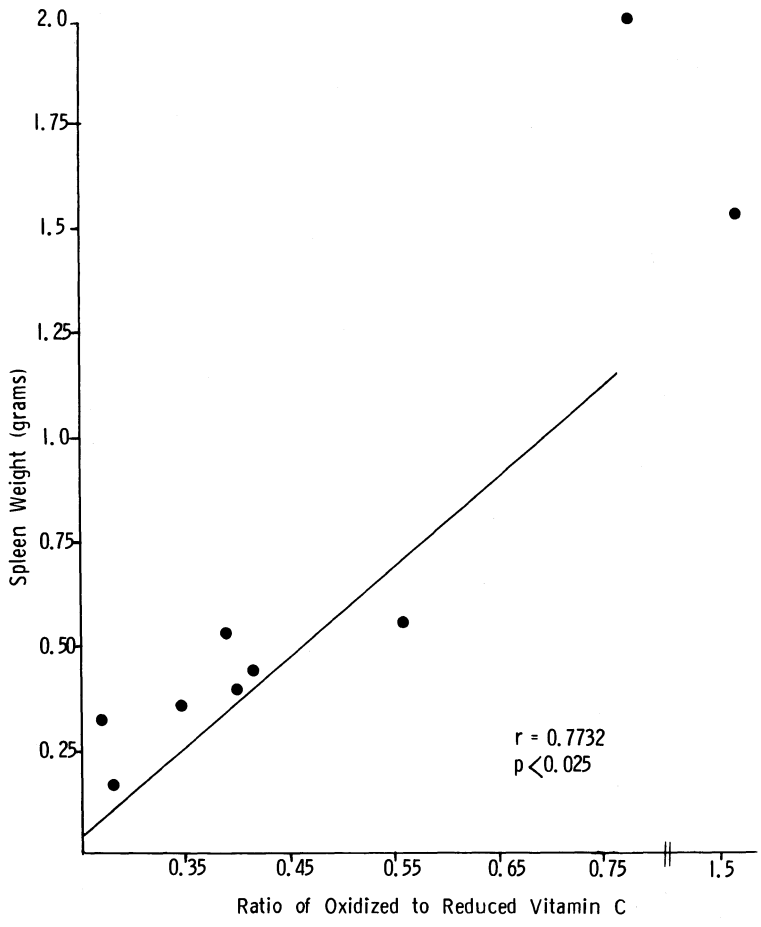

FIGURE 4. Linear Regression Analysis of Spleen Weight as a Function of Oxidized to Reduced Vitamin C Ratios in the Spleen of Old, Autoimmune NZB Mice.

females. The results (Table III) indicate that long-surviving NZB females show lower spleen weights than the 16-20 month-old animals, suggesting that these very old mice did not develop autoimmunity. Of particular interest are the higher spleen vitamin C levels in these surviving mice, a result which may be interpreted in two ways: 1) the ability to store vitamin C in the spleen may reflect autoimmune progression and longevity in the NZB strain, or 2) the lack of autoimmunity (lower spleen weights) may reduce the extent of phagocytosis of antigen-antibody complexes in the spleen, which would then result in higher spleen vitamin C levels. The data presented above would support the latter hypothesis.

TABLE III

Effect of Survival to Very Old Age on Spleen Weights, Hematocrits, and Tissue Vitamin C Levels in NZB Female Mice

Age (mo.)	No. of mice	Spleen Wt. (g)±S.E.M.	Hct. ±S.E.M.	Vitamin C (mg%)± S.E.M. Spleen	Liver
16-20	10	0.2736±0.0356	36.1±2.51	31.1±2.27	20.8±1.21
		$p \ll 0.001$	$p > 0.5$	$p < 0.025$	$p < 0.025$
24-26	11	0.0910±0.0150	38.6±2.54	37.7±0.93	16.1±1.17

ASCORBIC ACID AND ANAPHYLAXIS

Ascorbic acid has been noted to be of benefit in the treatment of experimental anaphylaxis, particularly in combination with antihistamines. Dawson et al. (1966), for example, demonstrated that a combination of ascorbic acid (200 mg/kg body weight) and mepyramine, an antihistamine, given immediately prior to antigenic challenge, provided complete protection against mortality due to horse serum-induced anaphylaxis in the rat. Csaba and Toth (1971) have induced anaphylaxis in the mouse by ovalbumin, and have reported a 30% reduction in mortality by the combined treatment with ascorbic acid and Suprastin, an antihistamine, administered intraperitoneally 20 minutes before challenge. In humans, ascorbic acid has been reported to prevent histamine-induced airway constriction (Zuskin et al., 1973) as well as to inhibit flax dust-induced bronchoconstriction (Valic and Zuskin, 1973). Such studies have suggested a protective role for ascorbic acid in experimental anaphylaxis. We have therefore examined the effect of ascorbic acid treatment without antihistamines on mortality due to ovalbumin-induced anaphylactic shock in BALB/c female mice, and have employed a dietary regimen of ascorbic acid in contrast to the massive ascorbate injections of the earlier reported studies.

BALB/c female mice, purchased from Jackson Laboratory, Bar Harbor, Main, were exposed to a regimen of 250 mg% L-ascorbic acid in the drinking water beginning at 6 weeks of age, while control animals were given tap water only. This treatment continued throughout the course of the experiment. The ascorbic acid-containing water was administered in brown glass bottles and changed every other day in order to avoid oxidation of the ascorbic acid. All mice received standard laboratory chow (Ralston-Purina Co., Saint Louis, Mo.) ad libitum. Anaphylaxis was induced essentially

by the method of Csaba and Toth (1971). Beginning at 30 weeks of age, mice were primed with 7 weekly intraperitoneal injections of 2.0 mg ovalbumin (Grade V, Sigma Chemical Co., Saint Louis, Mo.) in 0.25 ml of physiologic saline. At 40 weeks of age, severe anaphylactic shock was induced following intravenous injection by tail vein of 10 mg ovalbumin in a total of 0.50 ml physiologic saline. Total vitamin C levels were determined as described above. Histamine was separated from tissue homogenates by the method of Picatoste et al. (1977), and assayed fluorimetrically by the method of Weir (1974).

The effect of supplementary ascorbic acid on anaphylaxis-induced mortality is shown in Table IV. A 44% reduction in mortality was noted for the ascorbic acid-treated mice compared to tap water-treated controls ($p<0.01$). This is the first indication that dietary ascorbic acid, without antihistamines, may provide protection against anaphylaxis-induced mortality.

TABLE IV

Effect of Supplementary Ascorbic Acid on Anaphylaxis-Induced Mortality in BALB/c Female Mice

Group	No. of mice	Percent mortality	P value
Tap water controls	19	74	
			<0.01
Vitamin C-treated	20	30	

The effect of ovalbumin immunization on tissue vitamin C levels is shown in Table V. Immunized non-anaphylactic mice (group B) and immunized anaphylactic mice (group C) showed significantly lower vitamin C levels in the lung, spleen, and liver compared to non-immunized tap water-treated mice (group A). As might be expected, vitamin C-treated mice dying of anaphylaxis (group D) displayed significantly elevated tissue vitamin C levels compared to anaphylactic tap water-treated mice (group C).

The effect of ovalbumin immunization on tissue histamine levels is shown in Table VI. Immunized non-anaphylactic mice (group B) showed significantly elevated histamine levels in lung and spleen, but not in liver, compared to non-immunized tap water controls (group A). Immunized mice, dying from anaphylaxis

TABLE V

Effect of Ovalbumin-Induced Anaphylaxis on Tissue Vitamin C Levels in BALB/c Female Mice

Group	Treatment	No. of mice	Vitamin C (mg/100 g wet weight)±S.E.M. Lung	Spleen	Liver
A	Unimmunized controls	8	30.0±1.02	45.6±1.95	27.2±1.14
			p<0.005	p<0.001	p<0.001
B	Immunized non-anaphylactic controls[a]	3	26.5±0.87	38.4±0.15	14.7±0.56
C	Immunized anaphylactic controls[b]	8	23.9±0.76	34.4±0.77	13.9±0.35
			p<0.001	p<0.001	p<0.001
D	Immunized anaphylactic vitamin C-treated[b]	6	35.8±0.48	41.4±0.62	28.7±1.79

[a] Mice were primed three times with 2.0 mg ovalbumin in 0.25 ml of physiologic saline.

[b] Mice were taken for vitamin C determinations immediately following death from anaphylaxis, and were assayed for total vitamin C as described in the text.

(group C) showed increases in lung and spleen histamine while liver histamine was unaffected. Anaphylactic mice, which had been maintained on a long-term regimen of vitamin C in the drinking water (group D) showed lessened increases in lung and spleen histamine levels. Further, we have observed (Figure 5) a significant inverse relationship between lung histamine levels and lung vitamin C levels in tap water-treated mice dying from anaphylaxis ($r= -0.896$, $p<0.005$). A significant inverse relationship between spleen histamine and spleen vitamin C levels was also noted in these mice (Figure 6) ($r= -0.911$, $p<0.005$). No such relationship was observed in the liver.

The elevation of lung histamine levels during both immunization and immunization with anaphylaxis would be expected since histamine is the primary mediator of anaphylactic shock. However, the observed increase in spleen histamine in both groups is unique, and might suggest the spleen as a target organ for anaphylaxis in the mouse.

The depression in tissue vitamin C levels following anaphylaxis is a novel finding, and would suggest that ascorbic

TABLE VI

Effect of Ovalbumin-Induced Anaphylaxis on Tissue Histamine in BALB/c Female Mice

Group	Treatment	No. of mice	Histamine (μg/g wet weight) ± S.E.M. Lung	Spleen	Liver
A	Unimmunized controls	8	0.93±0.07	0.82±0.07	0.18±0.03
			$p<0.005$	$p<0.001$	$p>0.5$
B	Immunized non-anaphylactic controls [a]	3	1.45±0.24	1.63±0.16	0.14±0.08
C	Immunized anaphylactic controls [b]	8	2.88±0.13	3.89±0.23	0.22±0.02
			$p<0.005$	$p<0.005$	$p>0.5$
D	Immunized anaphylactic vitamin C-treated [b]	6	1.96±0.26	3.02±0.17	0.24±0.05

[a] Mice were primed three times with 2.0 mg ovalbumin in 0.25 ml of physiologic saline.

[b] Mice were taken for histamine determinations immediately following death from anaphylaxis, and were assayed as described in the text.

acid is depleted during anaphylaxis. Ascorbic acid is also utilized during immunization without anaphylaxis, since significant decreases in tissue vitamin C levels were also observed in group B mice primed three times with ovalbumin. This is reminiscent of the vitamin C diminution observed (Table I) following immunization with SRBC's, and provides further support for the concept of phagocytosis-induced, free radical-mediated decreases in vitamin C levels.

Ascorbic acid-treated mice exhibited significantly lower lung and spleen histamine values following death from anaphylaxis; this might suggest a protective effect of vitamin C due to lowering of histamine concentrations in the target organs of the mouse. A possible mechanism of action of ascorbic acid is suggested by the observations of Imango (1955) that ascorbic acid cleaves the imidazole ring of histamine *in vitro* and of Chatterjee et al. (1975) of similar *in vitro* destruction of histamine by ascorbic acid. These results, combined with the protective effect of supplementary ascorbic acid on mortality following anaphylactic shock, suggest the possible use of vitamin C in the treatment of human immediate-type hypersensitivities.

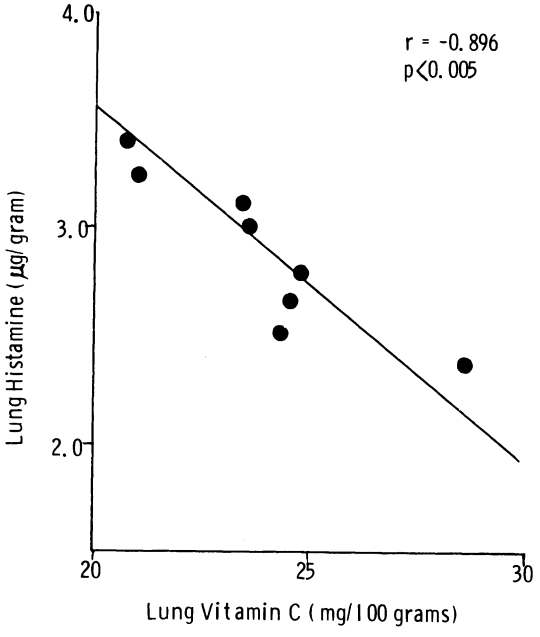

FIGURE 5. Relationship Between Histamine and Vitamin C Levels in the Lungs of Mice Dying from Anaphylaxis.

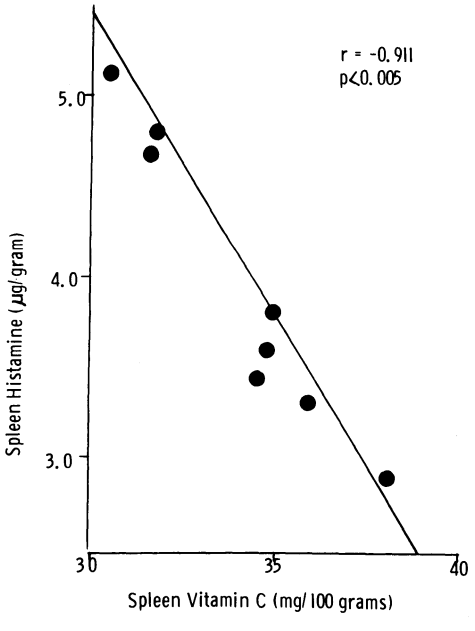

FIGURE 6. Relationship Between Histamine and Vitamin C Levels in the Spleens of Mice Dying from Anaphylaxis.

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