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NEW CLINICAL APPLICATIONS IN IMMUNOLOGY, LIPID METABOLISM AND CANCER

Edited by A. Hanck

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Foreword

International Symposia on vitamin C in Brazil have almost become a tradition. After the extremely successful Symposia held in Rio de Janeiro in 1976 and 1978, a Third International Symposium on vitamin C was held in São Paulo from the 8th to the 10th September 1980 under the auspices of the Academia Brasileira de Medicina Militar and the Medical Faculty of the State University of São Paulo. The Symposium was chaired by Linus Pauling, Bras Itapaci Magalhães and Alfred Hanck.

Central topics were new findings on the role of vitamin C in immunology, in cancer and in lipid metabolism. Workshops produced fruitful discussions of new findings and current aspects, aiming at a better understanding of the role of vitamin C in health and disease. These may help in decision-making for further research and practical application of our present knowledge.

It is hoped that this book will reach as broad an audience as did these on previous Symposia, adding to the medical knowledge about vitamin C, not least for the benefit of the patients.

A. Hanck

Introduction

L. PAULING

Linus Pauling Institute of Science and Medicine, Palo Alto, USA

First I want to thank the organizers of this Third International Symposium on vitamin C for having organized it and also for having invited my wife and me to come here. I think that this is a very important symposium. From my reading of the pre-prints of the papers, I see that valuable results have been obtained during the last two years and these are going to be presented by the participants from several countries who are present to participate in the symposium. I am especially pleased to note that there is now an emphasis on the use of larger amounts of vitamin C. I remember that Prof. A. SZENT-GYÖRGYI over 40 years ago, in the 1930s, said that there are two questions that we should ask about vitamin C and other vitamins – a few years after he had first extracted ascorbic acid from plants and animals, and this was identified as vitamin C – he said that we should study the vitamins to find out how much of an intake is needed to prevent the corresponding deficiency diseases. Then he went on to say that we should also determine the amounts of these substances that will put people in the best of health and he was very enthusiastic about the possibility of an astonishing improvement in the health of human beings by the proper use of these powerful substances. Nevertheless not much happened in this direction for several decades. In the 1940s there were reports in the medical literature, especially by physicians in Germany, to the effect that patients with various diseases, including cancer, showed a remarkable benefit when they were given what seemed to be large doses of vitamin C – perhaps 2 g a day, with also an increased intake of vitamin A. These studies were essentially ignored. Investigation of vitamin C in large doses in relation to disease did not become popular and the use of the vitamin in this way was not continued.

Other studies that were carried out and were said to use massive doses of vitamin C usually involved amounts of 200 mg per day, which we consider to be pretty small. I think myself that 200 mg a day should be considered the minimum recommended intake. The fact that it takes about this amount to saturate the process of tubular re-absorption of the vitamin in the kidneys, bringing it back from the glomerular filtrate into the blood is, it seems to me, strong evidence that amounts of vitamin up to this level are essential for good health. So I recommend that the national committees that make the recommendations about the intake of vitamins raise the level from 20, 30 or 50, 60 mg a day to 150–200 mg a day as the minimum recommended intake. Then of course we have the question as to what the optimum intake is: the amount which puts people in the best of health.

Back in 1949, there came, so far as I am aware, the first significant recommendation that the optimum intake be set at around 1,000 or 2,000 mg a day, 20 times or

40 times the usual intake. This was by Dr. GEOFFREY BOURNE in England when he was a member of the committee that made recommendations about vitamins. His arguments were ignored for many years. I am pleased to note that on the program during these 3 days there are reports of a number of investigations of the value of vitamin C in relation to the immune mechanisms of protection against disease. I think that it is likely that the value of a high intake of vitamin C results for the most part from the potentiation of these immune mechanisms. There are probably other ways in which the high intake of vitamin C also has value, but this is, I believe, the most important one. I am also pleased that there is to be discussion of the value of vitamin C in heart disease as well as cancer and other diseases. The principal cause of death is heart disease, and while usually death by heart disease does not involve so much suffering for the person himself or members of the family as death by cancer, nevertheless it has to be considered as one of the most important diseases to be investigated by researchers.

The fact that vitamin C operates largely by potentiating the body's natural protective mechanisms explains why the high intake of vitamin C has been reported over and over again to have value for the control of, and treatment of, many different diseases. This is understandable. I know that it is customary to say that if someone stands up and says that he knows something that is good for you, no matter what's wrong with you, he must be a quack! But I believe that vitamin C is such a substance and that it is reasonable to support that view in that something that makes the body stronger and more able to resist disease can be understood to be good for you, no matter what is wrong with you. Even stresses of various sorts are to some extent controlled by a large intake of vitamin C, as was emphasized by Professor TERENCE ANDERSON of Toronto a few years ago.

I am looking forward to hearing the papers that are going to be presented at this Symposium and I am glad to be here together with all of you.

Thank you.

Key words:
Viral infection
Cancer therapy
Malignancy
Interferon formation
Immune response
Leukemia development
Asthma
Hay fever
Serum sickness

The Multifactorial Role of Vitamin C in Health and Disease

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Summary: It is well established that nutrition can have a strong influence on resistance to disease. Nutritional deficiencies have been shown, in general, to lead to increased susceptibility to infections. Nutritional excesses, on the other hand, do not necessarily result in increased resistance; although in the case of ascorbic acid, there have been several reports demonstrating its efficacy with respect to some kinds of viral infections and disease processes. The role that vitamin C may play in its putative participation against viral infection, and possibly against malignancy, is the focus of the present report.

The studies to be discussed indicate that ascorbic acid enhances interferon formation and also plays a role in modulating the immune response. While the inclusion of ascorbic acid in the drinking water was observed to be without effect on the humoral antibody response, there was significantly increased cell-mediated immune reactivity. Ascorbic acid administration appeared to provide some protective effect under natural conditions of spontaneous leukemia development, as was similarly the case under experimental conditions of exogenous viral induction. Ascorbic acid administration also proved effective in reducing the mortality and prolonging the survival of anaphylactically-induced mice. Animals on a vitamin C regimen showed significantly higher tissue values of total vitamin C and lower tissue histamine levels, suggesting a protective effect of vitamin C due to lowering of histamine concentrations. Conceivably, vitamin C could be of benefit in the treatment of immediate-type hypersensitivities, such as asthma, hayfever, and serum sickness, and perhaps in other situations where histamine levels are elevated. Immunization with sheep erythrocytes was noted to diminish the levels of total vitamin C in phagocytic organs, namely spleen and liver, while serum and thymus values were unaffected by such treatment. Further, chronic phagocytosis of antigen-antibody complexes in the spleens of the autoimmune NZB mouse was observed to reduce total vitamin C levels as well as increasing the ratio of oxidized to reduced vitamin C, suggesting that events associated with phagocytosis result in oxidation and loss of the ascorbic acid fraction of vitamin C. Additionally, because of its enhancement of interferon production and its associated activation of cytotoxic macrophages, vitamin C should be further investigated as a feasible adjunct in some forms of cancer therapy.

It is well established that nutrition can have a strong influence on resistance to disease. Nutritional deficiencies have been shown, in general, to lead to increased susceptibility to infections. While early studies did not differentiate between resistance and immunity, in the main these studies tended to indicate that malnutrition and infection were mutually aggravating within a wide range of nutritive states. There is substantial evidence, now, that prolonged malnutrition is suppressive of the immune response through depression of cell-mediated immunity and humoral antibody production, as well as through diminished phagocytic activity [1] and non-specific resistance factors.

Nutritional excesses, on the other hand, do not necessarily result in increased resistance; although in the case of ascorbic acid, there have been several reports demonstrating its efficacy with respect to some kinds of viral infections and disease processes [16]. The role that vitamin C may play in its putative participation against viral infection, and possibly against malignancy, is the focus of the present report.

The mechanism of the proposed protective action of ascorbic acid against viral infections, as for example, the common cold in humans, is not well understood [17]. The possibility of its role in viral inactivation, as immunologic enhancer, and in interferon stimulation has been variously postulated. With regard to the latter, induced or exogenously administered interferon has been reported to bring about transient remission in patients with acute leukemia, and to retard metastases and, in some cases, even to effect regression in such tumors as osteogenic sarcoma, multiple myeloma, and breast cancer.

Interferons are a class of glycoprotein (protein plus carbohydrate) of a molecular weight of about 20,000, capable of inhibiting virus replication in vertebrate cells. They are of cellular origin and are induced in the human and animal host in response to infections by many viruses and other intracellular parasites. A fairly wide range of materials including synthetic polynucleotides can stimulate the production of interferon. The antiviral effect does not result from the direct inactivation of the virus or from non-specific toxic effects on the cells. Rather it reacts with cells to induce the formations of a new intracellular substance, a polypeptide or small protein. In some manner, interferon protects susceptible host cells from translating viral-coded messages, yet does not interfere with the cell's translation of messages into host cell proteins.

Interferon produced against one virus will inhibit the replication of a wide spectrum of other viruses. However, interferons are generally animal species specific in their range of antiviral activity, so that only interferons induced in human cells will be effective in treatment of humans. There are, in general, two main types of interferon: Virus (type 1) interferon, also called fibroblast and leukocyte interferon, induced by viruses and synthetic polynucleotides: and Immune (type 2) interferon, a lymphokine, induced by exposing macrophages and T-cells to mitogens and antigens. These differ antigenically and in pH stability.

The present sources of human interferon for research and therapy are mainly of three types: leukocyte interferon produced from donor blood buffy coats, fibroblast

interferon induced from the cultured fibroblasts of infant foreskins, and immune interferon produced from a lymphoblastoid Burkitt's lymphoma strain, the latter's use limited by their tumor origin. More recently, it has been reported that the human gene for interferon has been isolated and cloned by scientists working for the Biogen company. The Biogen group has apparently succeeded in inserting the human interferon gene into the *Escherichia coli* genome. Some of the bacteria interpreting its passenger's genetic blueprint as part of their own reproductive process proceed to make proteins with antiviral characteristics of human interferon.

In our own studies [18] we have observed a relationship between the interferon response of an animal and its leukemic susceptibility. Serum interferon assays of three inbred strains of mice, NZB, BALB/c, and C57B1/6, which had been inoculated with RAUSCHER leukemia virus (RLV), showed that interferon levels were lower for the NZB than the BALB/c and highest for the C57B1/6. Interestingly, and perhaps significantly, the BALB/c evinced less leukemia development than the NZB, while the C57B1/6 were refractory to RLV infection (Fig. 1).

In some of our early experiments we were able to demonstrate an enhancing effect of a vitamin C regimen on interferon induction by the RAUSCHER virus [19, 21] or by synthetic polynucleotides [20]. BALB/c mice on an L-ascorbic acid regimen of 250 mg% in their drinking water for three months showed significant increases in serum interferon levels over ordinary water controls following RLV administration [21]. Subsequently the protective effect of vitamin C in RAUSCHER viral leukemogenesis has been investigated. Here, while leukemia symptoms were considerably diminished in the animals on vitamin C, some disease development was evident (Fig. 2).

In succeeding studies the leukemic incidence was followed in strains of mice, such as the AKR and SJL, which are known to develop lymphomas spontaneously, thus obviating experimental manipulation by the investigator. The AKR strain is prone to

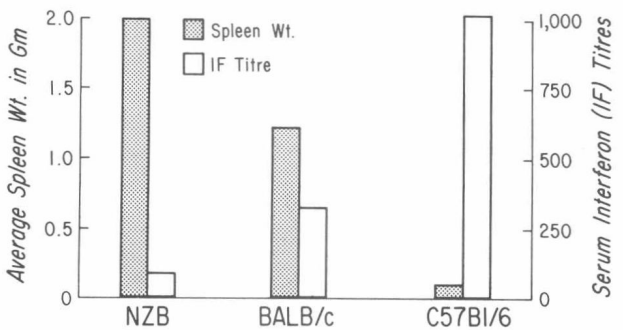

Fig. 1: Average spleen weights of 4–6 NZB, BALB/c, and C57B1/6 strain mice inoculated i. v. with 0.2 ml of a 0.1% spleen cell extract of RAUSCHER leukemia virus at 6 weeks of age and harvested 28 days later–compared to serum interferon titres of a similar number of each of these strains which were induced following RAUSCHER virus infection. This figure contrasts interferon inducibility by RAUSCHER leukemia virus (RLV) of these inbred mouse strains with their leukemogenic susceptibility to the virus and suggests, but does not prove, and inverse relationship between these.

spontaneous lymphocytic leukemia of established viral etiology from the age of 8 months. The SJL strain spontaneously develops a very high and early incidence of reticulum-cell neoplasms (90%) usually from the age of 8 months. While a viral causation has not been established in the case of the SJL, the fact that some attempts to reproduce the diseases by cell-free extracts have succeeded speaks in favor of such an etiology [28]. In these experiments, ascorbic acid administration would appear to have provided some protective effect under natural conditions of spontaneous leu-

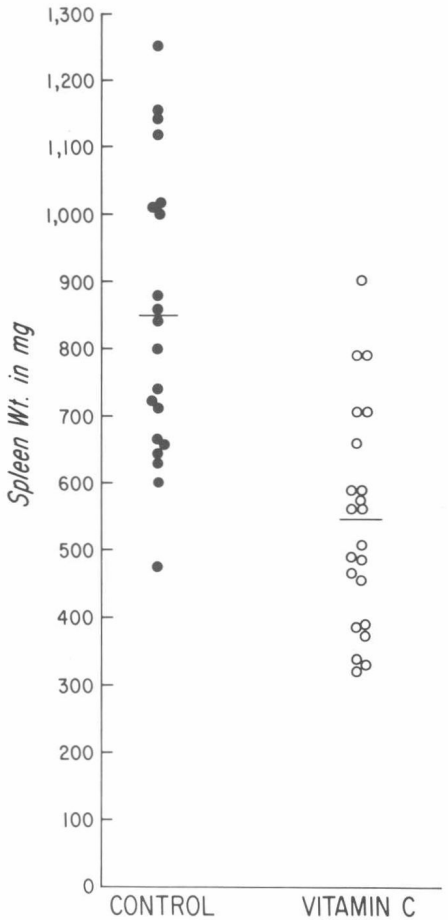

Fig. 2: DBA/2J female mice were started on a regimen of 250 mg% L-ascorbic acid in the drinking water at 7 weeks of age. They were inoculated i. p. with a 1% spleen cell extract of RLV two weeks later. The ascorbic acid regimen was continued to termination of the experiment 3 weeks later. Points represent spleen weights of individual mice, the horizontal bars indicating mean spleen weights for each of the two groups. Splenomegaly was noted to be considerably diminished in many of the animals on vitamin C, and the difference was found to be significant at the $p<0.001$ level. However, it should be noted that some disease development (spleen weight >0.25 g) was evident in all the RLV-infected mice.

kemia development. AKR mice on vitamin C, for example, showed an overall reduced rate of leukemia development in contrast to the controls (Fig. 3). In the SJL strain, however, a marked protective effect on leukemia development was observed (Fig. 4). SJL mice treated with vitamin C exhibited a 50% survival time of approxi-

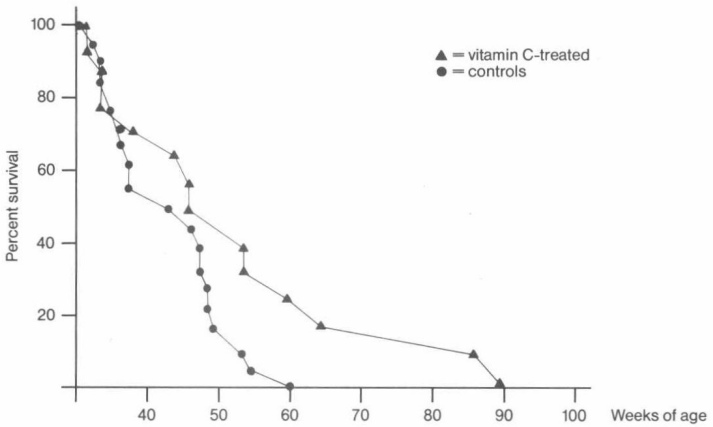

Fig. 3: Effect of L-ascorbic acid on the survival of leukemic AKR/J male mice. Mice were placed on an ascorbic acid regimen of 250 mg% in the drinking water at 6 weeks of age until termination of the experiment. Controls received tap water. Only those mice having thymus weights over 100 mg and spleen weights above 250 mg were regarded as having died of leukemia. The mortality rate of the two groups is seen to be similar up to 50 weeks of age, with a distinctly retarded rate of leukemia development and death after that time.

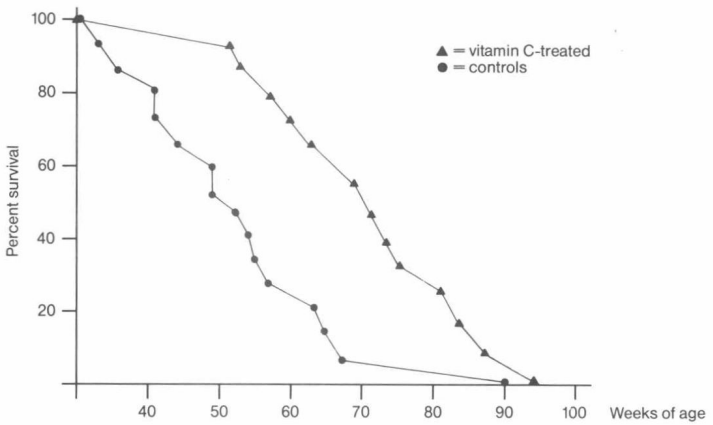

Fig. 4: Effect of L-ascorbic acid on the survival of leukemic SJL/J male mice. Mice were placed on an ascorbic acid regimen of 250 mg% in the drinking water at 6 weeks of age until termination of the experiment. Controls received tap water. Only those mice having spleen weights over 250 mg were considered as having died from leukemic involvement. A significant protective effect of vitamin C supplementation was noted with a 50% survival time of 72 weeks in the vitamin C-treated group as compared to 48 weeks in the controls.

mately 70 weeks, while control mice were noted to exhibit a 50% survival time of 50 weeks. In these studies, all mice which died of causes other than leukemia were excluded from all calculations. The protective effect of ascorbic acid supplements against murine leukemia are less striking than the therapeutic effect of ascorbic acid in human cancer, as reported by PAULING and CAMERON [4]. This difference is clearly related to the ability of mice, and most mammals, to synthesize ascorbic acid. Therefore, animals which lack the capacity to produce ascorbic acid would be expected to exhibit a greater pharmacological effect than those capable of its production.

Vitamin C appears to affect various aspects of the immune response. Its high concentration in leucocytes and its rapid utilization during infection has suggested a role for the vitamin in the immune response. There is evidence from animal experimentation that ascorbic acid is involved in a number of neutrophil functions, including chemotactic responses, phagocytosis, hexose monophosphate shunt activity, myeloperoxidase function, and cyclic GMP levels [12]. Ascorbic acid also appears to affect other facets of the immune response, including delayed hypersensitivity reactions, immediate hypersensitivity, and monocyte-macrophage reactivity, as recently reviewed [13].

Suggestive evidence that interferon might play a regulatory role in the immune process led us to investigate whether ascorbic acid could exert a modulating effect on the immune response [22]. In this regard, there did not appear to be any significant effect of vitamin C supplementation on the responsiveness of the bone marrow-derived lymphocyte (B-cell) as determined by humoral antibody responses of BALB/c mice to sheep red blood cells and similarly to bacterial lipopolysaccharide. Our observations are in agreement with most studies on vitamin C and antibody production, in which vitamin C deficiency and supplementary vitamin C were noted to exhibit no effect on antibody production [13]. However, there was a significantly increased cell-mediated immune response as determined by increased T-lymphocyte responses to concanavalin A. The cell-mediated immune processes (CMI) in which T-lymphocytes participate include the release of a variety of soluble effector molecules or lymphokines which are probably responsible for effecting CMI. For example, entrapment of macrophages by such lymphokines as macrophage inhibiting factor (MIF) and heightened activation by macrophage activation factor (MAF) result in more ready ingestion and degradation of infectious agents. Thus, the cell-mediated immune response is considered to provide a mechanism for enabling the infected host to rid itself of viruses and other intracellular parasites. Conceivably, this protective role of vitamin C could be expanded to include some forms of cancer [23].

In point of fact, there is some evidence now that interferon may itself be a factor responsible for the activation of macrophages to a cytotoxic state, and thus may be implicated in destruction of tumor cells [1, 10]. In this context, a macrophage population selectively killing neoplastic cells may have considerable importance for surveillance, since such a cell population might eliminate neoplastic cells as they arise

in the affected host [24]. Thus, the reported enhancement of macrophage function by vitamin C supplements assumes greater importance, and provides novel areas for investigations on the possible role of ascorbic acid in neoplasia.

More recent experiments in our laboratory have addressed the role ascorbic acid may play in the immediate-type hypersensitivity reactions. In studies of ovalbumin-induced anaphylaxis in the mouse, a dietary regimen of 250 mg% L-ascorbic acid in the drinking water was observed to reduce mortality significantly (Table I). BALB/c female mice 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 was maintained throughout the course of the experiment. Anaphylaxis was induced essentially by the method of CSABA and TOTH [7]. Beginning at 30 weeks of age, mice were primed with 7 weekly intraperitoneal (i. p.) 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 (i. v.) injection by tail vein of 10 mg ovalbumin in a total of 0.50 ml physiologic saline. Total vitamin C levels (ascorbic acid, dehydroascorbic acid, and 2,3-diketogulonic acid) were determined as previously described [25], and histamine was separated by phosphorylated cellulose chromatography and assayed fluorometrically [26].

The effect of supplementary ascorbic acid on anaphylaxis-induced mortality is shown in Table I. A 44% reduction in mortality was noted for the ascorbic acid-treated mice compared to tap water-treated controls ($p<0.01$). Although previous investigations have noted a synergistic effect of ascorbic acid in combination with antihistamines in the reduction of anaphylaxis-induced mortality [7, 8], the present report is the first indication that dietary ascorbic acid, alone, may provide protection against anaphylaxis-induced mortality.

The effect of ovalbumin immunization on tissue vitamin C levels is shown in Table II. Immunized non-anaphylactic mice (group B) and immunized anaphylactic mice (group C) showed significantly lowered 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 tap water-treated mice which died from anaphylaxis (group C).

The effect of ovalbumin immunization on tissue histamine levels is shown in Table III. Immunized non-anaphylactic mice (group B) showed significantly elevated

Tab. I: 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	< 0.01

Tab. II: 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 p<0.005	45.6 ± 1.95 p<0.001	27.2 ± 1.14 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 p<0.001	34.4 ± 0.77 p<0.001	13.9 ± 0.35 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.

Tab. III: 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 p<0.005	0.82 ± 0.07 p<0.001	0.18 ± 0.03 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 p<0.005	3.89 ± 0.23 p<0.005	0.22 ± 0.02 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.

histamine levels in lung and spleen, but not in liver, compared to non-immunized tap water controls (group A). Immunized mice, dying from anaphylaxis (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 ascorbic acid in the drinking water (group D) showed lessened increases in lung and spleen histamine levels. Further, a significant inverse relationship was observed (Fig. 5) 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 (Fig. 6) ($r = -0.911$, $p<0.005$). No such relationship was observed in the liver.