

PREVENTION OF EXPERIMENTAL ALLERGIC
ENCEPHALOMYELITIS (EAE) BY VITAMIN
C DEPRIVATION*

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The occurrence of a marked lipemia in the sera of many paralyzed guinea pigs after the induction of experimental allergic encephalomyelitis (EAE) led the present authors to investigate the variation in plasma free fatty acid concentrations during this disease (1). Because of the well known effect of fasting on fatty acid mobilization, and because paralyzed animals do not eat well, it was planned to utilize vitamin C deprivation as a means of ruling out this non-specific effect of illness on plasma free fatty acid concentrations. Mueller and Cardon (2) had shown that scorbutic guinea pigs were unable to mobilize plasma free fatty acids in response to the fasting stimulus. If elevations had been noted in scorbutic animals with EAE, it could be stated that such elevations were not secondary to fasting. In attempting to carry out this experiment, we observed that animals on a scorbutic diet did not succumb to the disease, whereas normal diet controls, receiving the same challenge, were severely affected.

This observation led to an investigation of the effect of vitamin C deficiency on induction of EAE and an attempt to analyze this phenomenon with regard to its quantitative aspects. It was possible to demonstrate that the greater the challenge, the less effective the deficient diet was in preventing the disease. Suppression of disease by the C-deficient diet depended on the nutritional deficiency being established in the animal prior to the development of EAE. The dietary deficiency also inhibited the development of delayed hypersensitivity to tuberculin protein as evidenced by skin reactivity.

EXPERIMENTAL

Animals.—Disease-free, mixed color guinea pigs, bred in a closed colony in the Animal Production Section at the National Institutes of Health, were used for all of the experiments

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described. The animals were young males varying in weight from 250 to 500 gm depending upon the individual experiment. All animals were housed in large general purpose cages, 6 to a cage, on a bed of sawdust and cedar shavings. They were obtained from the stock colony 1 week prior to encephalitogenic challenge and maintained on the diet specified.

Scorbutic animals were fed a vitamin C-deficient diet supplied by Nutritional Biochemical Corporation. In the first experiment they were injected intraperitoneally with 0.1 ml physiological saline daily, whereas normal diet controls received this diet plus 50 mg ascorbic acid in 0.1 ml saline daily intraperitoneally. All other "normal diet controls" were given a regular stock diet for guinea pigs (NIH formula A), plus a daily supplement of greens (cabbage and kale) to provide an adequate supply of ascorbic acid.

In one experiment, designed to test the effect of weight loss *per se* on disease induction, the guinea pigs were maintained on the normal diet regimen for 7 days following cord-adjutant injection and then given greens only. The latter provided vitamin C but the caloric intake was so low that the animals lost weight at approximately the same rate as the scorbutic animals after 2 weeks on the deficient diet.

Induction of Experimental Allergic Encephalomyelitis (EAE).—The disease was induced in the guinea pigs by one intracutaneous injection of 0.1 ml of the following emulsion: 4 mg lyophilized bovine cord, 1 ml phenol-saline (0.5 per cent phenol-0.9 per cent NaCl), 1 ml melted aquaphor (Duke Laboratories) and 2 ml mineral oil (Fisher Scientific Co.) containing 4 mg ground *Mycobacterium butyricum* (Difco).

The mixture was homogenized in a glass homogenizer, incubated 1 hour at 60°C and then rehomogenized immediately before injection. 0.1 ml of the warm emulsion was injected into a shaved area over the sternum. This mixture containing both bovine cord and killed mycobacteria is referred to as "CNS injection."

Animals were weighed daily and examined for signs of neurological damage, such as hind leg weakness or paralysis, inability to right themselves after being placed on their backs, fecal impaction, tremors, etc. Some of these signs were difficult to assess in scorbutic guinea pigs because of the generalized weakness, swollen feet, etc., associated with advanced scurvy. Complete paralysis of the hind extremities was the only clinical sign which was associated with EAE and not with scurvy.

The disease index used for a quantitative measure of disease severity was the same as that described by Alvord and Kies (3). The scale of 0 (no disease) to 10 (maximum response) is based on independently evaluated clinical and histological evidence of disease. The median day of onset (*i.e.*, the day on which half or more of the animals became paralyzed) was also determined and is another indication of severity of EAE (4).

Most of the experiments were terminated 21 days after the CNS injection was given, 28 days after the scorbutic animals had been placed on the special diet. Any variations from this time schedule are described under the individual experiments.

Determination of Plasma 17-Hydroxycorticosteroids.—17-hydroxycorticosteroids were determined by the method of Silber and Porter (5) on plasma samples collected from the severed vessels of the neck through silicone-coated funnels into silicone-coated test tubes containing 3 drops of heparin (100 mg/ml).¹

RESULTS

Table I shows the results of vitamin C deprivation on EAE. Both control and scorbutic animals were observed for 21 days after EAE induction (28 days after beginning the ascorbic acid-deficient diet). Eight of the 12 control

¹ We are indebted to Mr. Silas Jackson of the National Cancer Institute for the 17-hydroxycorticosteroid determinations.

animals became paralyzed and showed histological changes typical of EAE. The disease index of this group was 7.2, and the mean plasma FFA level was 0.73 ± 0.33 mEq/liter, significantly elevated above normal ($p < 0.01$). Two of the 9 available plasma samples (3 animals died of sudden severe paralysis before blood could be obtained) were lipemic, and their FFA levels were above 1.00 mEq/liter.

TABLE I
Prevention of EAE by Vitamin C Deficiency

	N	Incidence and severity of EAE		
		Clinical	Histological	Disease index
Control diet.....	12	8/12	11/12	7.2
Scorbutic diet.....	11	0/11	1/11	0.3*

* $p < 0.001$.

TABLE II
Effect of Different Dosages of Bovine Spinal Cord and Mycobacteria on Inhibition of EAE by Vitamin C-Deficient Diet

Bovine cord/ mycobacteria	Diet	N	Severity of EAE	
			Disease index	Day of onset (median)
<i>mg</i>				
0.1/0.1	Control	12	7.2	15
"	Scorbutic	11	0.3*	21
1.0/0.1	Control	6	7.0	12
"	Scorbutic	12	1.5*	21
1.0/1.0	Control	6	8.2	13
"	Scorbutic	6	5.2	18

* $p < 0.001$.

The scorbutic group, on the other hand, showed no paralysis, lipemia, or elevated FFA. Only one of the 11 animals showed histological evidence of EAE and this was in the spinal cord only. The mean disease index of the scorbutic guinea pigs was 0.3.

In order to test the effectiveness of the protection against EAE afforded by vitamin C deficiency, stronger encephalitogenic vaccines were prepared by increasing the dose of bovine cord and mycobacteria (Table II). Even with massive doses of cord and mycobacteria, scurvy afforded significant although incomplete protection against EAE. In each instance the scorbutic group had

a lower disease index and a significantly later date of onset than its corresponding control group.

In Table III the degree of protection against EAE by scurvy is seen to be related to the nutritional status of the animals at the time the CNS injection is given. If the diet was begun 1 week prior to inoculation, the protection was almost complete: the disease index of the scorbutic animals was 0.8, and none of the animals became paralyzed. When the diet and inoculation were initiated simultaneously, the disease index of the vitamin C deficient group rose to 3.7, and 7 of 12 guinea pigs were paralyzed. When the ascorbic acid-deficient diet was begun 1 week after inoculation, there was no difference in susceptibility to EAE between normal and scorbutic animals. Although not yet quantitated, the completeness of the vitamin C deficiency is important; when a diet was

TABLE III
Degree of EAE Inhibition Related to Duration of Scorbutogenic Diet

Scorbutogenic diet initiated	N	EAE severity (disease index)	Tuberculin sensitivity (per cent PPD positive)
1 wk. before EAE injection	6	0.2*	0
With EAE injection	12	3.8	0
1 wk. after EAE injection	6	5.2	67
Normal diet control	6	6.3	100

* $p < 0.001$.

used which was inadequate but not completely deficient in vitamin C, no protection from EAE could be demonstrated.

In the same experiment the proportion of animals having a positive tuberculin skin test to PPD 14 days after inoculation with killed mycobacteria was also found to be related to the duration of the scorbutic diet prior to the skin test. None of the animals which had been on the diet for 14 or 21 days showed a positive skin reaction to PPD. Four of 6 animals were sensitive to PPD after 7 days of vitamin C deficiency, and all of those on the normal diet were sensitive to PPD.

Fig. 1 further demonstrates the effect of scurvy on development of skin sensitivity to tuberculin. Guinea pigs were tested with PPD 11, 15, and 21 days after injection with 0.1 mg of dried *Mycobacterium butyricum* in an aquaphor-water-oil emulsion (18, 22, and 28 days after beginning the scorbutogenic diet). A normal non-sensitized control group was also included. The sensitized animals on a normal diet showed progressively increasing response to PPD, whereas the scorbutic and unsensitized control groups showed no significant response to PPD.

Fig. 2 shows the effect of scurvy on a second inoculation of killed mycobacteria. Both normal and scorbutic animals were inoculated intracutaneously

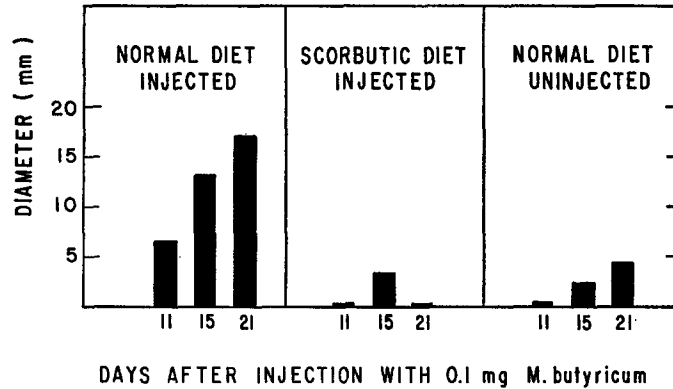


FIG. 1. The effect of vitamin C-deficient diet on tuberculin skin hypersensitivity at 11, 15, and 21 days after injection with 0.1 mg *Mycobacterium butyricum* in water-in-oil emulsion. Twenty-four hours after 5 µg PPD was injected intradermally, test site was observed and diameter of indurated area recorded. The vitamin C-deficient diet was begun 1 week prior to *Mycobacterium butyricum* injection.

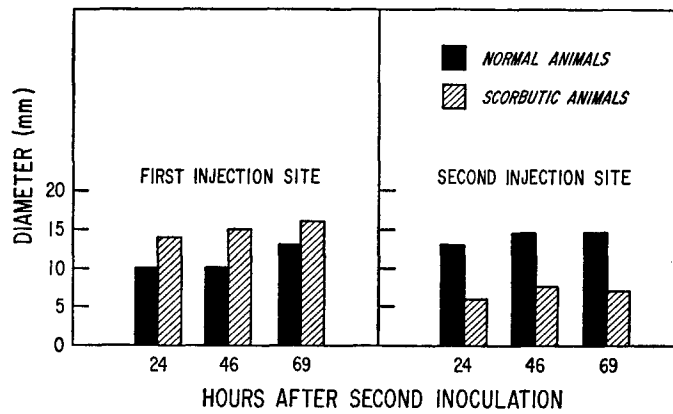


FIG. 2. Effect of vitamin C deficiency on primary and secondary injection site response to 0.1 mg intradermal injection of *Mycobacterium butyricum* in water-in-oil emulsion. Vitamin C-deficient diet was begun 1 week prior to the first injection. Diameter of indurated areas was measured 24, 46, and 69 hours after second injection.

with Freund's adjuvant and reinoculated 14 days later with the same mixture in a different site. The scorbutic animals showed a significantly decreased area of response at the second injection site ($p < 0.01$). Histologically, this site was characterized by the presence of marked hemorrhage and slight increase in

proliferation of capillaries in the scorbutic animals, but there was no difference in degree of edema or leukocytic infiltration as compared to the second injection

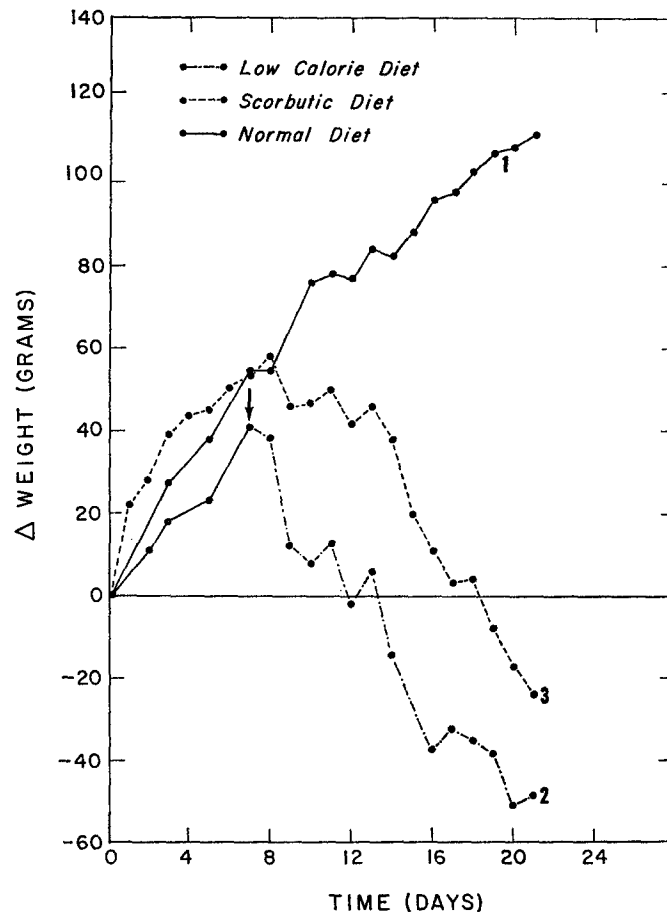


FIG. 3. Mean weight curves of 3 groups of guinea pigs injected on day 0 with 0.1 mg lyophilized bovine cord and 0.1 mg *Mycobacterium butyricum* in water-in-oil emulsion. Group 1 was fed normal diet. Group 2 was fed normal diet 7 days and then given low calorie diet consisting of cabbage and kale only. Group 3 was fed vitamin C-deficient diet which was begun 1 week prior to CNS-adjutant injection.

site in the normal animals. At this time the site of first inoculation was slightly larger in the scorbutic animals than in the normal animals, but histologically no significant differences could be detected.

As a control for cachexia, which becomes increasingly severe after the 18th day of scurvy, 6 animals were deprived of all food except greens 7 days after CNS inoculation. Fig. 3 shows the weight curves of 3 groups of animals chal-

lenged with CNS vaccine at day 0: Curve 1, normal diet throughout the course of the experiment; Curve 2, normal diet for first 7 days and then low calorie diet, and Curve 3, scorbutogenic diet beginning 1 week before challenge (see Table III). The "starved" animals had no significant difference in susceptibility to EAE from the normally fed animals. Their disease indices were 7.5 and 6.3 respectively, in contrast to the absence of EAE in the scorbutic animals (disease index 0.2). All animals of the first 2 groups were sensitive to PPD 14 days after CNS inoculation, whereas the scorbutic animals were not (Table III).

The effect of vitamin C deprivation and caloric deprivation on the plasma 17-hydroxycorticosteroids was determined in connection with their effects on development of EAE and PPD sensitivity. The results are shown in Table IV. It is obvious that the increase in plasma 17-hydroxycorticosteroids noted in

TABLE IV
Comparative Effects of Inanition with and without Vitamin C Deprivation on Plasma 17-Hydroxycorticosteroids, PPD Sensitivity, and EAE Susceptibility

Dietary status	Plasma 17-hydroxycorticosteroids (Mean \pm s.d.)	PPD reactivity	EAE susceptibility
	<i>$\mu\text{g./100 ml}$</i>		
Normal.....	72 \pm 15	+	+
Vitamin C deprivation.....	207 \pm 78*	-	-
Caloric deprivation†.....	310 \pm 62*	+	+

* $p < 0.001$.

† Supplemented with greens.

the scorbutic animals is not responsible for the other effects observed because "starvation" also resulted in markedly elevated 17-hydroxycorticosteroid levels and did not suppress development of EAE or PPD sensitivity.

Finally, daily supplements of 50 mg of ascorbic acid were given intraperitoneally to each of 9 animals which had failed to develop EAE for 21 days after 28 days on the scorbutic diet. Although none of these animals developed any clinical signs of EAE after complete recovery from scurvy up to 50 days after their initial inoculation with CNS, 7 of the 9 animals were found to have recovered their PPD sensitivity 50 days after the initial CNS-adjuvant injection. Reinjection with a second inoculation of CNS in complete adjuvant 50 days after the first inoculation also failed to induce EAE in any of these 9 animals, possibly because of the prior sensitization to the mycobacterium (6) and/or neural tissue (7).

DISCUSSION

From these data it would appear that deprivation of vitamin C markedly inhibits the development of EAE. The protection afforded by scurvy is in-

versely proportional to the strength of the encephalitogenic vaccine used and directly proportional to the severity of the vitamin C deficiency attained. PPD sensitivity and the local reaction to a second adjuvant inoculation are also inhibited by scurvy. However, when vitamin C is restored to the scorbutic animals 21 days after inoculation with CNS and mycobacteria, the animals recover tuberculin sensitivity but do not develop encephalomyelitis. The protection offered by scurvy thus seems to be different from the suppression of EAE noted with 6-mercaptopurine (8). The fact that tuberculin sensitivity is also suppressed by scurvy is consistent with the autoimmune theory of EAE development. The recovery of tuberculin sensitivity following restitution of vitamin C, however, may indicate only that the mechanisms underlying tuberculin sensitivity are much cruder than those underlying the development of EAE, which depends upon a rather fine balance between concentrations of CNS and mycobacteria (4, 9).

In scurvy, in addition to the well known inhibition of collagen formation as reviewed by Hunt (10), a number of other effects have been reported, including threefold or greater increase in plasma (11, 12) and urinary (13) 17-hydroxycorticosteroids. Although cortisone has been reported to suppress EAE in monkeys (14), it does not prevent the development of EAE in guinea pigs (15). This latter observation is consistent with the results reported here that endogenous elevations of plasma 17-hydroxycorticosteroids are not correlated with suppression of EAE.

In addition, there is decreased lymphocyte synthesis during scurvy (16). Although EAE is generally considered to be due to sensitization of white blood cells, there has been some difference of opinion regarding the cell type involved. Thus in reference 17 Waksman discusses the histiocyte (page 166) Campbell, the plasma cell (page 168) and Chase, the lymphocyte (page 348). Far greater decreases in white blood cells have been observed after x-irradiation than during scurvy, but x-irradiation does not prevent EAE in the guinea pig (15).

The failure of animals to develop EAE in the absence of certain essential nutrients has also been observed by Schneider, Lee, and Olitsky (18) who found that the susceptibility of an inbred mouse strain to EAE induction was less on a purified diet than on fox chow, bread, and milk. The susceptibility was partially restored by supplementation of the purified diet with folic acid, vitamin B₁₂, and biotin. It should be noted that the purified diet they used was adequate for maintenance of growth and well being of the animals, even though it would not permit development of EAE without supplementation.

The fact that suppression of EAE in the scorbutic animals was not a non-specific result of general inanition was shown by the fact that severe caloric deprivation in the presence of adequate vitamin C intake did not suppress induction of EAE. Partial fasting results in increased plasma 17-hydroxycorticosteroids, just as vitamin C deprivation and x-irradiation do, but lack

of vitamin C is the only one of the 3 states which suppresses development of EAE.

Thus, one is left with the conclusion that although the role of vitamin C in this disease reaction is obscure, the vitamin nevertheless does exert some influence. Previous studies on the effects of vitamin C on immunological reactions have led to contradictory conclusions. Some of these papers have been reviewed by Boyden (19) who concluded from the reports cited that vitamin C probably was concerned in some way with the allergic reaction, but no definite hypothesis regarding its effect was formulated.

SUMMARY

Scorbutic guinea pigs injected with CNS and mycobacterium to induce experimental allergic encephalomyelitis (EAE) showed no clear-cut neurological signs and failed to show histological evidence of central nervous system damage. The degree of protection afforded by vitamin C deprivation was related directly to the duration of the scorbutogenic diet and inversely to the strength of the CNS challenge.

Vitamin C deprivation also abolished tuberculin sensitivity as measured by the PPD skin reaction. Upon restoration of vitamin C, the animals recovered their sensitivity to PPD but did not develop EAE.

It was further demonstrated that these effects of vitamin C deprivation were not related to inanition or to the endogenous levels of 17-hydroxycorticosteroids.

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