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EFFECT OF VARIATION IN ENDOGENOUS LEVELS OF ASCORBIC ACID ON THE *in vitro* IMMUNOLOGICAL RELEASE OF HISTAMINE AND SLOW REACTING SUBSTANCE OF ANAPHYLAXIS FROM ACTIVELY SENSITIZED GUINEA-PIG LUNG FRAGMENTS

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1 The *in vitro* immunological release of histamine and slow reacting substance of anaphylaxis (SRS-A) from actively sensitized guinea-pig lung fragments was greatly reduced when the animals were maintained on an ascorbic acid-deficient diet. Excessive dietary levels of ascorbic acid did not increase mediator release above normal levels.

2 Restoration of ascorbic acid in the diet of scorbutic guinea-pigs restored *in vitro* immunological histamine to normal levels.

3 Variation in dietary levels of ascorbic acid had no effect on lung histamine content.

4 The lung ascorbic acid content was proportional to the dietary intake. Approximately 60% of the total lung ascorbic acid was removed by the process of chopping and washing of the tissue. This relationship was independent of dietary intake.

5 The results indicate that the immunological release of mediators of inflammation from guinea-pig lung is dependent on adequate endogenous levels of ascorbic acid.

Introduction

In vivo studies have shown that variations in the local concentrations of ascorbic acid alter airway responses to exogenous constrictor agents. High doses of ascorbic acid protect sensitized guinea-pigs maintained on a normal diet against the effects of antigen aerosols (Dawson & West, 1965a; Guirgis, 1965). Actively sensitized scorbutic guinea-pigs are less sensitive to antigen aerosols than guinea-pigs maintained on normal diets (Dawson & West, 1965a). Restoration of normal dietary levels of ascorbic acid restored the normal response to antigen aerosol. These data suggest that the immunological response is dependent on adequate endogenous levels of ascorbic acid. The present study tests this hypothesis. Data on the effect of variation in endogenous levels of ascorbic acid on the *in vitro* release of histamine and slow reacting substance of anaphylaxis (SRS-A) are given. Some of these results were presented at the Meeting of the Federation of American Societies for Experimental Biology (Hitchcock, 1977).

Methods

The animals used in this study were adult male albino guinea-pigs (Hartley strain, 250 g). They were maintained on an ascorbic acid-free diet throughout the experiment. The diet, which was in pellet form and prepared by Ralston Purina Co., Richmond, Indiana, U.S.A., contained 20% protein, 5% fat, 75% carbohydrate and was free of cane molasses, wheat, alfalfa and animal fat. Dietary exposure to ascorbic acid was via the drinking water. The animals were divided into three groups having a calculated daily ascorbic acid intake of 0.5, 10 or 100 mg per day. This was achieved by exposing the animals to tap water containing respectively 0.005, 0.1 or 1.0 mg/ml ascorbic acid and assuming the average daily intake of water was 100 ml per animal. Animals maintained on 0.005 mg/ml showed signs of scurvy after 21 days at which point they received 0.5 mg ascorbic acid per day by oral injection. Seven days after the beginning of exposure to specific amounts of ascorbic acid, the animals were actively sensitized with a single injection (i.p.) of 10 mg egg albumin in 1 ml of 0.9% w/v NaCl solution. *In vitro* mediator release was determined 28 days later.

In a second series of experiments, animals which had been maintained on 0.5 mg per day ascorbic acid were given 10 mg ascorbic acid per day 28 days after sensitization. Mediator release was determined 37

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days after sensitization. Lungs from control groups of animals maintained on either 0.5 mg or 10 mg per day throughout the period were also examined for mediator release 37 days after sensitization.

Determination of mediator release

The lungs were removed and placed in Tyrode buffer pH 7.4, the composition of which was (mM): NaCl 136.7, KCl 2.7, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, NaH_2PO_4 0.36 and glucose 5.8. The lung tissue was chopped, incubated, and mediator release determined as described previously (Hitchcock, 1978). Briefly, weighed aliquots (100 mg) of chopped lung fragments were incubated in Tyrode solution at 37°C in a final volume of 5 ml. Following a 5 min equilibration period, the sensitized lung was challenged with egg albumin (0.2 to 40 μg/ml final concentration). After 15 min, the incubates were filtered and the amount of histamine released determined in the filtrate spectrofluorimetrically (Shore, Burkhalter & Cohn, 1959). The data are expressed in terms of the percentage of total histamine released per 100 mg chopped lung in 15 min. Total histamine was determined as described previously (Hitchcock, 1978). SRS-A in the filtrate was determined by bioassay on the guinea-pig ileum in the presence of atropine sulphate (3.4 μg/ml), mepyramine maleate (0.34 μg/ml) and methysergide bimaleate (0.1 μg/ml). FPL 55712 (0.1 μg/ml) was used to block the contractions attributed to SRS-A. The contractions were compared with a laboratory standard of crude SRS-A and are expressed in arbitrary units. The standard was obtained by incubating sensitized chopped lung (5 g) with egg albumin (5 mg) in a final volume of 25 ml at 37°C for 30 min. The incubate was filtered and aliquots of the filtrate were frozen at −70°C. One unit of SRS-A was contained in 50 μl of filtrate. The same SRS-A standard was used in all the experiments described. The concentration of ascorbic acid was determined on 100 mg samples of whole and chopped lung by the spectrophotometric method of Roe & Kuether (1943).

Statistics

Student's unpaired t test was used to determine statistical significance. P values less than 0.05 were considered statistically significant.

Drugs

The following chemicals and drugs were used: histamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.), egg albumin 5 times crystallized (Nutritional Biochemicals, Cleveland, Oh., U.S.A.), and L-ascorbic acid (Fisher Scientific Co., Fairlawn, N.J., U.S.A.). Sodium 7-[3(4-acetyl-3-hydroxy-2-propyl phenoxy)-2-hydroxy propoxy]-4 oxo-8-propyl-4H-1-benzopyran-2-carboxylate (FPL 55712, Fisons Ltd., Loughborough, Leics.) was kindly supplied by the manufacturer.

Stock solutions of all chemicals were freshly prepared each day. FPL 55712 was prepared in distilled water (1 mg/ml) and diluted with Tyrode solution. All other chemicals and drugs were dissolved in Tyrode solution.

Results

The data in Table 1 demonstrate the relationships between the level of dietary ascorbic acid, ascorbic acid concentrations in whole lung and chopped lung fragments, and total lung histamine. In a segment of whole lung, ascorbic acid content increased with the level of ascorbic acid in the diet. By comparison, male guinea-pigs maintained in this laboratory on standard chow had lung ascorbic acid levels of 90% of those found in guinea-pigs maintained on 10 mg per day ascorbic acid. The ascorbic acid content of chopped lung fragments was between 38 and 46% of that present in the sample of whole lung and this relationship appeared to be independent of the dietary intake of ascorbic acid. Thus approximately 60% of the ascorbic acid in whole lung can be removed by the

Table 1 Guinea-pig lung ascorbic acid and histamine content at different dietary levels of ascorbic acid

	Ascorbic acid concentration				
Diet (mg/day)	*Whole lung* (μg/100 mg)	*Chopped lung* (μg/100 mg)	$\frac{\text{Chopped}}{\text{Whole}} \times 100$	n	*Total histamine* (μg/100 mg lung)
0.5	4.075 ± 0.555	1.709 ± 0.197	42.76 ± 5.05	14	1.402 ± 0.28
10.0	27.654 ± 1.255	12.605 ± 0.999	46.46 ± 4.10	10	1.678 ± 0.23
100.0	40.289 ± 1.690*	15.227 ± 0.651*	38.38 ± 1.83	14	1.494 ± 0.16

* Significantly greater than 10 mg/day: $P < 0.001$.

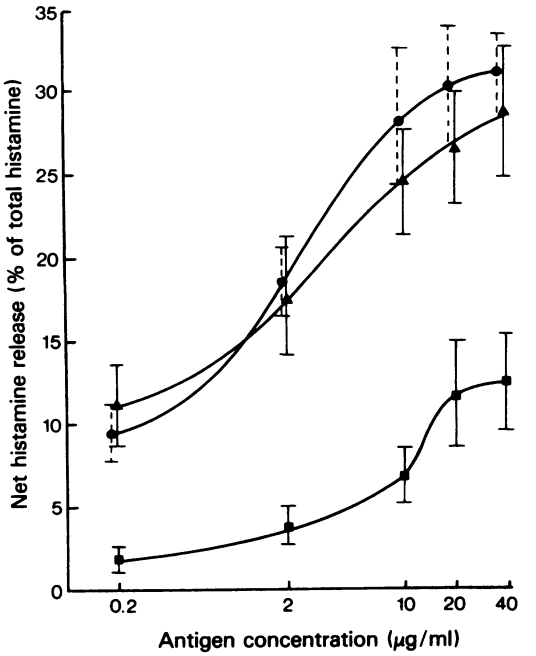

Figure 1 Effect of variation in dietary ascorbic acid level on *in vitro* release of histamine from actively sensitized chopped guinea-pig lung. Dose-response curves of % of total histamine released as a function of antigen concentration. Dietary ascorbic acid levels were as follows: (■) 0.5; (●) 10.0; (▲) 100.0 mg/day. Vertical bars represent s.e. mean. Each dose-response curve is the average of 10 experiments.

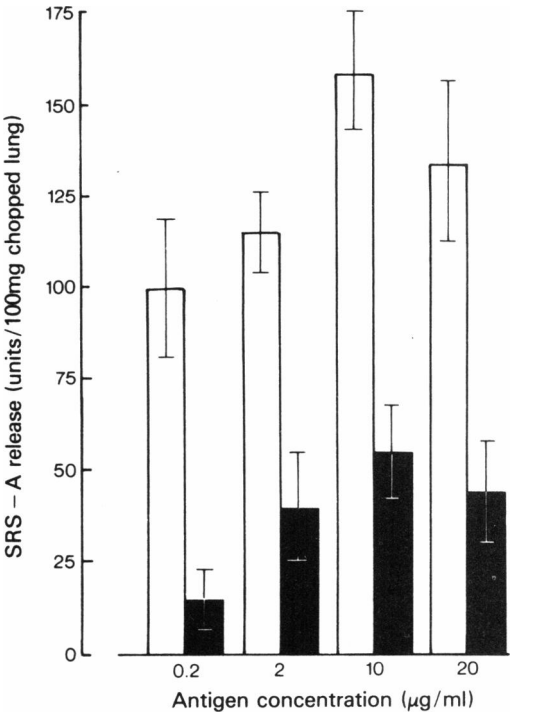

Figure 2 Effect of variation in dietary ascorbic acid level on *in vitro* release of slow reacting substance of anaphylaxis (SRS-A) from actively sensitized chopped guinea-pig lung. Open and solid columns of the histogram represent SRS-A release from chopped lung from animals maintained on 10 and 0.5 mg ascorbic acid per day, respectively. Vertical bars represent s.e. mean. Data are the average of 10 experiments for each level of ascorbic acid.

process of chopping and washing. Previous studies have demonstrated that the histamine content of chopped lung is approximately 90% of that present in whole lung (Hitchcock, unpublished observations). Total histamine present in chopped lung was not significantly different at the three levels of dietary ascorbic acid (Table 1). Thus major differences in endogenous ascorbic acid levels do not appear to have an effect on total lung histamine. The total histamine in chopped lung fragments from animals maintained on a synthetic diet was within the 50th percentile of the normal range of the total histamine in lungs from animals maintained on standard chow (Hitchcock, 1978).

Dose-response curves of *in vitro* histamine release versus antigen concentration for chopped lung obtained from animals maintained on the three levels of dietary ascorbic acid are shown in Figure 1. Spontaneous histamine release was not significantly different between the three groups of animals and averaged 3.2% of the total histamine. A significantly smaller percentage of the total lung histamine was released from the lungs from animals maintained on 0.5 mg ascorbic acid per day compared with lungs from animals maintained on 10 and 100 mg per day (Figure 1). Differences between the proportion of histamine released from lungs from animals maintained on 10 and 100 mg per day were not statistically significant. Incorporation of L-ascorbic acid (1 to 3 μg/ml) into the incubates containing lung from animals maintained on 0.5 mg per day did not increase histamine release to normal values.

The effect of differences in dietary ascorbic acid levels on the release of SRS-A was similar to the effect on histamine release (Figure 2). The release of SRS-A from chopped lung containing 1.71 μg ascorbic acid per 100 mg lung averaged one third of that released by chopped lung containing 12.60 μg ascorbic acid per 100 mg lung. Figure 2 also demonstrates that the

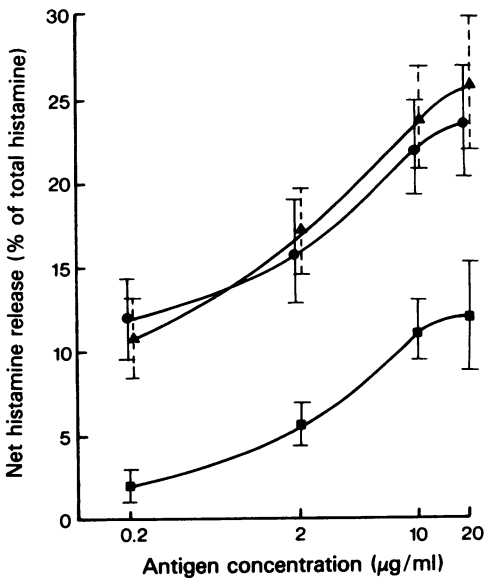

Figure 3 Restoration of *in vitro* histamine release from actively sensitized chopped guinea-pig lung from deficient animals given normal dietary ascorbic acid levels. Details of experimental protocol given in Methods. Dietary ascorbic acid levels were as follows: deficient, (■) 0.5 mg/day for 37 days; normal, (▲) 10 mg/day for 37 days; reconstituted, (●) 0.5 mg/day for 28 days followed by 10 mg per day for 9 days. Vertical bars represent s.e. mean. Average ascorbic acid in chopped lung (μg/100 mg) was as follows: normal, 11.2; reconstituted, 10.0; deficient, 1.6.

release of SRS-A by 20 μg/ml antigen at both levels of ascorbic acid was less than that released by 10 μg/ml antigen. In similar experiments conducted on the lungs of animals maintained on standard chow, maximum SRS-A release was obtained at an antigen concentration of 40 μg/ml (Hitchcock, 1978).

Restoration of ascorbic acid (10 mg per day for nine days) in the drinking water of animals which had been sensitized and deficient (0.5 mg ascorbic acid per day for 28 days) resulted in the restoration of *in vitro* histamine release to normal levels (Figure 3). The lungs from animals maintained on 0.5 mg ascorbic acid per day throughout the 37 day period released a smaller proportion of the total histamine following antigen challenge compared with normal and reconstituted animals (Figure 3).

Discussion

The amounts of ascorbic acid used in this study were such that guinea pigs were supplied with deficient (0.5 mg per day), normal (10 mg per day) or excessive (100 mg per day) dietary concentrations of the vitamin. The results presented here support the hypothesis that an adequate endogenous level of ascorbic acid is necessary for the expression of the immunological response as defined by determination of mediator release *in vitro* and parallel the *in vivo* observations of Dawson & West (1965a). Mediator release was not affected by excessive dietary levels of ascorbic acid. Differences in the amount of histamine released in normal versus deficient animals could not be explained by differences in histamine content.

Adequate dietary ascorbic acid may be necessary for full sensitization. However, the speed with which mediator release in deficient animals was restored as a result of restoration of normal dietary ascorbic acid level suggests that this is not the case. A more likely explanation of the effect is the dependence on ascorbic acid of a factor which regulates immunological mediator release. This could be the metabolism of endogenous arachidonic acid. SRS-A from guinea-pig lung has been identified as a metabolite of arachidonic acid (Watanabe-Kohno & Parker, 1980). Immunological histamine release from guinea-pig lung is stimulated by indomethacin and other inhibitors of cyclo-oxygenase (Hitchcock, 1978). This indicates that products of this enzyme synthesized *de novo* as a consequence of the antigen-antibody reaction are important in regulating the amount of histamine released. Furthermore in the presence of inhibition of cyclo-oxygenase, prostaglandin $F_{2\alpha}$ stimulated the release of histamine from actively sensitized guinea-pig lung while prostaglandin E_2 inhibited the release (Hitchcock, 1978). Guinea-pig lung cyclo-oxygenase is a microsomal enzyme (Parkes & Eling, 1974) and guinea-pigs deficient in ascorbic acid are also deficient in microsomal protein and have reduced activity of a number of microsomal enzymes (Zannoni & Sato, 1975).

The ease with which part of the total lung ascorbic acid was removed during preparation of the tissue indicates that some of the vitamin is held extracellularly. The relationship between intracellular and extracellular lung ascorbic acid is independent of endogenous levels of the vitamin. Pulmonary lavage of intact rat lungs removes about 30% of the total lung ascorbic acid (Willis & Kratzing, 1974). Thus a proportion of the lung ascorbic acid is held in the fluid lining the air spaces. Furthermore, the lung may be an excretory organ for ascorbic acid. In addition to being necessary for immunological mediator release, ascorbic acid may have a role in the response of airway smooth muscle to inhaled agents. A single high dose of ascorbic acid protects guinea-pigs against the bronchoconstrictor effects of histamine, 5-hydroxytryptamine and antigen (Dawson & West, 1965a, b; Guirgis, 1965) by a direct effect on airway smooth muscle (Dawson & West, 1965b). In humans, a single

high dose of ascorbic acid protects against histamine aerosols (Zuskin, Lewis & Bouhuys, 1973) but not against antigen-induced asthma (Kordansky, Rosenthal & Norman, 1979). Histamine is not an important mediator in human allergic asthma but SRS-A is. Ascorbic acid may not antagonize the constriction due to SRS-A and other mediators. Thus ascorbic acid does not appear to be of therapeutic value in human allergic asthma.

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