Cancer Letters, 38 (1987) 73–85
Elsevier Scientific Publishers Ireland Ltd.

HYDROXYLAMINE EFFECTS ON CRYPTOGENIC NEOPLASM DEVELOPMENT IN C3H MICE

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(Received 27 July 1987)
(Revised version received 25 August 1987)
(Accepted 28 August 1987)

SUMMARY

The effect of administration of hydroxylamine (HA) to male and female mice was studied because of reports suggesting an anticarcinogenic effect and an enhancement of lifespan. In this study, two C3H sublines were used: the C3H/HeN which carries a germinal provirus of the mouse mammary tumor virus and the C3H/HeJ(+) which also carries the milk-transmitted exogenous virus. Lifetime administration of 10 mM HA in the drinking water resulted in a decrease in mammary neoplasm incidence in female C3H/HeN mice, but not in female C3H/HeJ(+) mice. Ovarian neoplasms and cysts were common in all groups, indicating ovarian dysfunction, but these were not affected by treatment. The incidences of other cryptogenic neoplasms found in controls in significant numbers, i.e. liver carcinomas, lymphomas, lung adenomas and adrenal cortex tumors were only marginally affected by the treatment. However, an increased incidence of vascular neoplasms of the spleen in hydroxylamine-treated female C3H/HeN mice and vascular neoplasms of the lymph nodes in hydroxylamine-treated male C3H/HeJ(+) mice indicated a subline-related action on the reticuloendothelial system. The survival of control mice was 35—58% at 2 years and this was not increased in either subline by hydroxylamine, which is interpreted to indicate that this antiioxidant does not increase lifespan of animals under conditions of maintenance that are adequate for good survival.

INTRODUCTION

The hydroxylated primary amine, hydroxylamine (HA), is formed as an intermediate by the enzymatic reduction of nitrates or nitrites or by the

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0304-3835/87/$03.50
Published and Printed in Ireland

oxidation of ammonia as a product of cell metabolism [4,14,27,37]. Exposure to exogenous HA can occur in the synthesis of nylon, in photography, from certain soaps and fatty acids, and from some tanning agents. HA is also formed in the body from drugs such as amphetamine, epinephrine and norepinephrine. It is moderately toxic to humans, animals and even plants, but only at concentrations substantially greater than those resulting from normal cell metabolism. HA is mutagenic in bacteria, fungi and viruses, [10,14,29] and clastogenic in plants and humans [14,25], but does not induce DNA repair in cultured rat hepatocytes [38].

In animal studies, Harman [15–18] reported that HA in the diet increased the lifespan and decreased the incidence of cryptogenic neoplasms in mice. Support for the potential of HA in cancer chemoprevention was provided by a study in which no neoplasms occurred in C3H/HeN mice kept on HA for as long as one year [40]. Also, the condition and appearance of the mice seemed to be better than that of untreated controls. Likewise, Evarts and Brown [7] found that HA treatment reduced the number of hyperplastic mammary nodules in mice treated with the agent from 6 weeks of age. HA has also been reported to have carcinostatic capabilities in several other experimental animal and cell culture studies [1,5,31]. When given prior to dimethylbenz[*a*]anthracene, HA protected against the injurious effects of the carcinogen on rat mammary tissue and when HA was given after the carcinogen there was a decrease in the incidence and number of tumors, as well as a lengthening of the expression time to tumor development [8].

The purpose of the present study was to determine the effect of lifetime administration of HA on neoplasm development and longevity in two mouse C3H sublines with high, but differing incidences of cryptogenic neoplasms due to the murine mammary tumor virus (MMTV). The study involved female C3H/HeN mice, which carry a dominantly expressed germinal provirus, Mtv-1, and develop a moderately high incidence of tumors appearing late in life and male and female C3H/HeJ(+) mice, which also carry the exogenous milk transmitted MMTV causing a high and early incidence of mammary tumors [28,34].

MATERIALS AND METHODS

Animals

Female C3H/HeN mice with the provirus (Experiment A) and male and female C3H/HeJ(+) mice with the exogenous virus (Experiment B) were obtained from the National Institutes of Health, Bethesda, MD at 4 weeks of age. They were housed, 5 animals/10½ × 19½ × 8″ polycarbonate cage. Bedding consisted of heat-treated hardwood chips; cages and food were changed three times a week. The basal diet, NIH-07 (Zeigler Bros., Gardners, PA), and tap water in bottles were freely available. Animals were housed in a temperature (21 °C ± 1 °C) and humidity (50 ± 10%) controlled room with a minimum of 14 air changes per hour. The rooms were maintained on a 12-h light/dark cycle.

The animal facility in which the mice were kept was an SPF facility which

operated on a clean/dirty corridor system. It was supervised by Jerald Silverman, DVM and was fully accredited by the American Association for the Accredition of Laboratory Animal Care. The care of animals conformed to the Guide for the Care and Use of Laboratory Animals (NIH 78-23).

Chemicals and administration

HA was obtained as HA sulfate from Sigma Chemical Company, St. Louis, MO. The concentration selected for chronic administration was derived from the toxicity studies and 1-year study done by Yamamoto et al. [40]. A solution of 10 mM HA was prepared by adding 16.4 g HA sulfate to 10 l of tap water and neutralizing it to pH 7 using KOH. Solutions were prepared fresh weekly and kept refrigerated until use. The treated mice were given HA solutions in brown glass bottles, while the control mice received plain drinking water.

Experimental design

Two separate experiments were conducted with C3H/HeN mice (Experiment A) and C3H/HeJ(+) mice (Experiment B). In Experiment A, 60 female C3H/HeN mice, Group 1, received no treatment while the HA solution was given to 40 females in Group 2. Experiment B consisted of C3H/HeJ(+) mice with 56 females, Group 3, maintained as untreated controls, 50 females, Group 4, given the HA solution, 50 males, Group 5, maintained as controls and 50 males, Group 6, given HA. The administration of all solutions was started when the mice were 6 weeks of age.

The exposures were made for 123 weeks in Experiment A, and for 105 weeks in Experiment B. The mice were observed regularly and all gross changes, including palpable mammary nodules were recorded. The animals were weighed weekly. They were killed when moribund or at termination. All animals were autopsied except those which were cannibalized or severely autolyzed. Non-autopsied mice were not included in the effective number of animals. At autopsy, all altered organs were described. The specimens and samples from all parenchymatous organs were fixed in buffered formalin. Slices were embedded in paraffin and slides prepared in the Histopathology Laboratory supervised by A. Rivenson, MD. Sections were stained with hematoxylin-eosin as well as other appropriate stains and subjected to microscopic study.

Statistical analysis was performed using the two-tailed Z test and Fischer's exact probability test.

RESULTS

Experiment A

In Experiment A, with C3H/HeN female mice, which carry the MMTV provirus, more of the animals that were given the HA drinking water (Group 2) survived for the full experimental period than did the corresponding untreated controls (Group 1), as shown in Fig. 1. All treated animals survived for the first

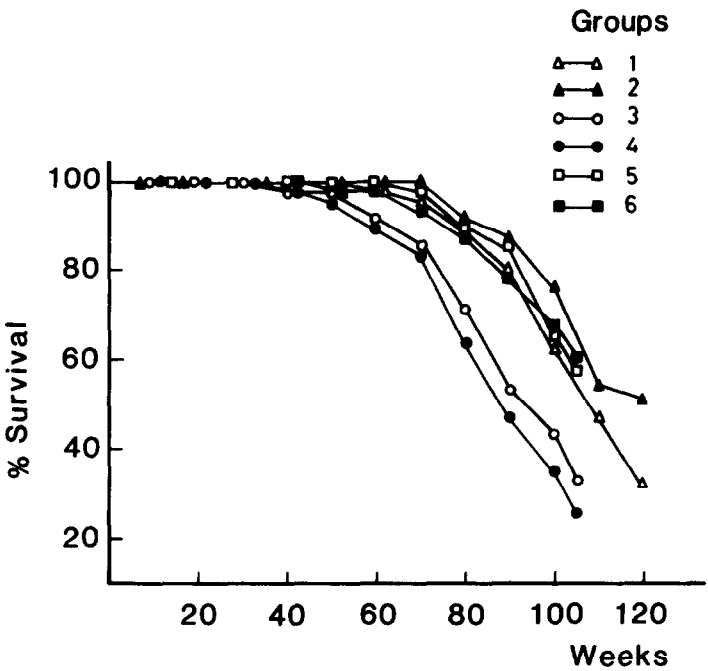

Fig. 1. Survival in weeks of mice treated with hydroxylamine and controls. 1 = C3H/HeN female controls; 2 = C3H/HeN female mice receiving HA for life; 3 = C3H/HeJ(+) female control mice; 4 = C3H/HeJ(+) female mice HA for life; 5 = C3H/HeJ(+) male control mice; and 6 = C3H/HeJ(+) male mice receiving HA for life.

70 weeks, with a survival improvement between 2 and 16% at different time intervals. The body weight of treated as well as untreated animals increased up to 50 weeks and remained stable thereafter. The average body weights of the animals in these two groups remained similar throughout the experiment (Fig. 2).

Experiment A, the occurrence of neoplasms in both control and treated mice was high (Table 1), both in regard to the number of animals with neoplasms and the number of neoplasms. Mammary carcinomas, lymphomas, lung adenomas, liver carcinomas and ovarian neoplasms were observed in controls, as well as HA-treated animals (Table 2). The mammary adenocarcinoma incidence was statistically significantly lower in HA-treated mice, 3 in 36 (8.3%), vs. 14 in 58 control animals (24%). In contrast, vascular neoplasms of the spleen occurred in 10 HA-treated animals, whereas none was found in the controls.

Experiment B

In Experiment B, which utilized C3H/HeJ(+) mice of both sexes, which carry the exogenous MMTV, the male mice had a higher survival than females and this was not affected by HA. The female mice which were given HA for life

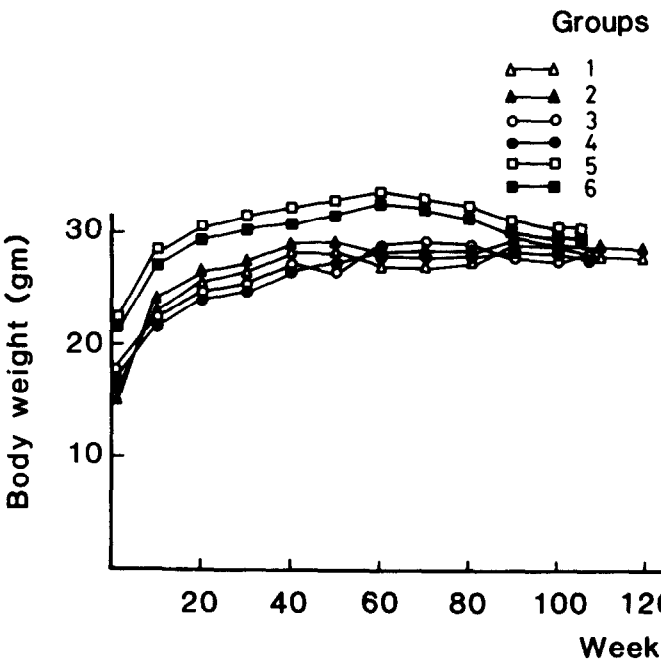

Fig. 2. Average weight of mice treated with hydroxylamine and controls. 1 = C3H/HeN female controls; 2 = C3H/HeN female mice receiving HA for life; 3 = C3H/HeJ(+) female control mice; 4 = C3H/HeJ(+) female mice receiving HA for life; 5 = C3H/HeJ(+) male control mice; and 6 = C3H/HeJ(+) male mice receiving HA for life.

(Group 4) had a slightly lower survival when compared to controls (Group 3), as shown in Fig. 1. Male mice had a higher average body weight than females (Fig. 2). HA administration caused a slight decrease in body weight in male C3H/HeJ(+) mice, while no effect was seen in females.

The occurrence of neoplasms in all groups was high, both with respect to the number of animals with neoplasms and the number of neoplasms (Table 1). Hepatocellular carcinomas, lung adenomas and lymphomas were observed in both sexes, although the incidence of liver carcinomas was greater in males (Table 2). In females, mammary and ovarian neoplasms were frequent. The incidence of mammary neoplasms was slightly higher in the HA-treated females. The number of vascular neoplasms of the spleen was not affected by HA administration, but the number of hemangiomas of lymph nodes was significantly higher in HA-treated males. The number of tumor-bearing treated male C3H/HeJ(+) animals was significantly higher than the corresponding control group, but the number of tumors in the group was not much different.

Comparisons of response as a function of subline and sex

Comparison of the specific types of neoplasms in the various groups revealed certain differences. The mammary neoplasm incidence in C3H/HeJ(+) females

TABLE 1

EFFECT OF HYDROXYLAMINE ON OCCURRENCE OF NEOPLASMS IN C3H MICE

Experiment	Group	Sex	Exposure	Strain	Total no. of			
					Initial mice	Effective mice	Mice with neoplasms	Neoplasms
A	1	F	None	C_3H/HeN	60	58	33	49
	2	F	HA	C_3H/HeN	40	36	27	40
B	3	F	None	C_3H/HeJ(+)	56	44	39	57
	4	F	HA	C_3H/HeJ(+)	50	50	42	65
	5	M	None	C_3H/HeJ(+)	50	44	25	39
	6	M	HA	C_3H/HeJ(+)	50	48	41*	54

*Statistically significant, $P < 0.01$.

TABLE 2

TOTAL NUMBER OF NEOPLASMS OF DIFFERENT TYPES IN C3H/HeN AND $C_3HEJ(+)$ MICE AND THE EFFECT OF HYDROXYLAMINE

Experiment group	Sex	Treatment	Mammary carcinoma	Hepatocellular carcinoma	Lung adenoma	Lymphoma	*Hemangioma*				Adrenal tumor	Ovary			
							Spleen	Lymph node	Liver	Other		Gran. cell tumor	Tubular Adenoma	Cyst.	Other
A 1	F	None	14*	6	7	4	–	–	1	–	2	4	6	33	5[a]
2	F	HA	3	8	3	5	10**	–	–	–	–	5	5	23	1[b]
B 3	F	None	32	4*	3	–	1	1	–	2	–	2	9	35	3[c]
4	F	HA	42	–	3	3	–	1	2	2	–	1	9	33	2[d]
5	M	None	–	19	6	–	2	5	1	–	4	–	–	–	2[e]
6	M	HA	–	21	2	5*	1	13*	–	1	10	–	–	–	1[f]

[a] 2 luteomas, 1 cervical polyp, 1 endometrial polyp., 1 uterine adenocarcinoma.

[b] 1 ear squamous cell carcinoma.

[c] 2 luteomas, 1 endometrial adenocarcinoma.

[d] 2 luteomas.

[e] 1 squamous carcinoma of the skin, 1 subcutaneous sarcoma.

[f] 1 skin keratoacanthoma.

*Statistically significantly higher, $P < 0.05$; **statistically significantly higher, $P < 0.01$.

was considerably greater than in the C3H/HeN females, reflecting the action of the exogenous MMTV. The incidence of mammary tumors was lowered significantly by HA treatment in C3H/HeN mice, but not in the C3H/HeJ(+). Histologically, the mammary neoplasms were composed of solid structures of cuboidal cells in all groups and no effect of treatment on morphology was apparent.

Ovarian neoplasms and cysts were common in females of both sublines regardless of treatment. The neoplasms were mainly granulosa cell neoplasms and tubular adenomas. Luteomas were less frequent. Cysts originating from the ovarian surface or from abortive follicles were present in all groups. Uterine endometrial hyperplasia was also common. Histologically, the neoplasms were similar in the different groups. Liver adenomas and hepatocellular carcinomas were common in most groups, with the highest number in males C3H/HeJ(+) mice. The number of hepatocellular neoplasms was lower in female (C3H/HeJ(+) mice given HA, but the difference was marginal.

Of special interest in this study were the hemangiomas of the spleen (Fig. 3) in HA-treated C3H/HeN mice. They consisted of dilated vascular channels lined by regular normal-appearing endothelial cells and filled with blood. Endothelial proliferation or atypicality were not observed nor was there evidence of malignant behavior such as invasion. In the spleens of C3H/HeN mice, connective tissue proliferation and fibrosis was marked in the HA-treated

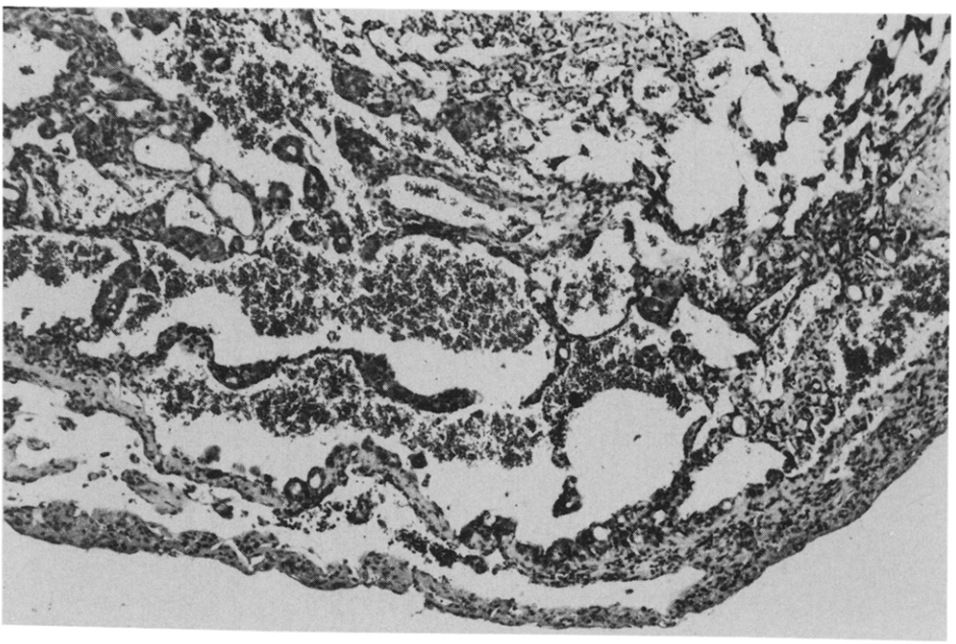

Fig. 3. Hemangioma of the spleen in a female C3H/HeN mouse treated with hydroxylamine. Widened vascular channels are lined by a regular endothelium. Orig. H&E × 360.

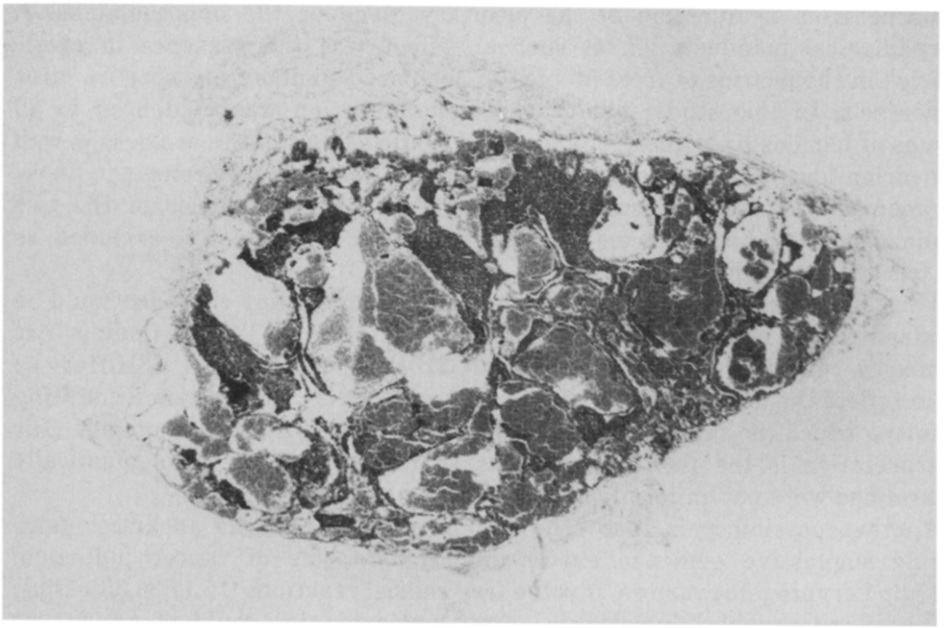

Fig. 4. Hemangioma of the lymph node in male C3H/HeJ(+) mouse treated with hydroxylamine. Blood-filled dilated spaces are lined by a regular endothelium. Orig. HE × 36.

group. Similar changes, but to a much lesser extent, were present in spleens of control mice. In the spleens of both treated and control C3H/HeJ(+) mice, some proliferation and fibrosis were present, but hemangiomas were few.

In the lymph nodes of C3H/HeJ(+) mice, vascular congestion was common, as was hemosiderin pigment accumulation from hemorrhages. Hemangiomas consisting of blood-filled, thin-walled dilated cystic spaces (Fig. 4), were found in control and HA-treated animals, with the incidence in HA-treated males being significantly higher. Lymphomas were also slightly increased in HA-treated C3H/HeJ(+)males.

DISCUSSION

This study examined the effect of lifetime administration of HA on neoplasm development in mice. A considerable reduction in mammary neoplasms incidence was observed in C3H/HeN female mice given HA for life, supporting the findings of Yamamoto et al. [40] and Evarts and Brown [7]. An inhibition, however, was not obtained in the C3H/HeJ(+) mice, which carry the exogenous milk-transmitted MMTV [28,34]. Several possibilities may be considered to explain this difference in response between sublines.

Evarts and Brown [7] suggested that the reduction of mouse mammary neoplasms in mice by HA was related to production of an hormonal imbalance

resulting from dysfunction of the pituitary gland or the hypothalamus. A morphological manifestation of such an effect was the presence of cystic follicles in the ovaries of treated mice as compared to numerous corpora lutea in controls. In this study, abnormal ovarian function was evidenced in all groups of females by the large numbers of cystic lesions in the ovaries, as well as ovarian and uterine neoplasms. Since there were no differences in these between treated and control mice or between treated females of the two sublines, hormonal imbalance cannot be implicated, but cannot be excluded, as the basis of the inhibition.

The C3H mice were chosen for study to determine if any evidence could be obtained for an effect of HA on expression of the MMTV. The finding that mammary tumors were reduced in the C3H/HeN, but not the C3H/HeJ(+) could reflect the fact that the provirus in the C3H/HeN has a weaker initiating activity, which is more easily modulated by HA. Perhaps against this interpretation is the finding that liver tumors, which are also genetically determined were not uniformly affected.

Another possibility is that HA may have functioned as an antioxidant. Highly suggestive evidence exists that some forms of cancer induction, including cryptogenic tumors, involve free radical reactions [15,17,33,35]. Thus, it is a reasonable hypothesis that antioxidants could function as anticarcinogenic agents [9,36] and the decreases in neoplasm incidences seen in the studies of HA reported by Harman [15–18] were thus ascribed to the antioxidant properties of the chemcial [16,17]. Other antioxidants such as butylated hydroxytoluene and similar agents have been effective in decreasing the incidence of carcinogen-induced neoplasms of the skin, lung, forestomach, liver and mammary gland [3,5,9,36,39]. This appears, however, to be due largely to alteration of carcinogen biotransformation [9,21,26,30]. If HA could exhibit cryptogenic neoplasm development through its antioxidant properties, in the present study, an effect on sites of low neoplasm incidence such as liver, lung and lymph nodes, might have been anticipated. This was not seen and, hence, the reduction of mammary neoplasms in the C3H/HeN mice is probably not due to such an action. Thus, there is no clear cut explanation for the effect of HA, but the availability of sublines with differing responses may permit further analysis.

This study may also be evaluated as a chronic carcinogenesis bioassay in mice since it involved lifetime exposure of males and females of one subline and females of another. HA did not show carcinogenic activity in most organs in the study, despite the fact that it is a mutagen in some systems, mainly prokaryotic [10,14,25,29]. Nevertheless, the number of hemangiomas was higher in HA-treated C3H/HeJ(+) males and C3H/HeN females. C3H mice are apparently prone to such lesions since they are known to have cystic lesions of the lymph nodes, referred to as mesenteric lymph node disease [6], which has certain similarities to hemangionas. The association of hemangionas with HA exposure is of interest since a structurally related group of substances, hydrazines, have been reported to produce neoplasms of the blood vessels in mice and hamsters

[32]. Symmetrical and unsymmetrical hydrazine derivatives produced angiosarcomas in various organs in a high percentage of animals [20,32]. The mechanism for this effect has not been elucidated. The basis for the effect of HA may be different, since HA is the parent compound of the series of arylhydroxylamines, thought to be reactive intermediates derived from arylamines through metabolism [22,27,37]. Several compounds which can form arylhydroxylamines, i.e. some aniline derivatives, have induced sarcomas of the spleen [12,13,19,37]. Some of these chemicals are known to cause methemoglobinemia [22,27]. HA has a number of effects on the hematopoietic system and erythrocytes [4]. The resulting damaged or senescent red cells may lead to splenic enlargement and eventually to splenic pathology, such as was observed in this study and that of Yamamoto et al. [40] in which HA induced splenomegaly and bone formation in the spleen.

The rather minimal adverse effects of HA with chronic administration suggest that it might be used safely for shorter periods. Hydrazine, which is more toxic, has been reported to be beneficial in the clinical management of cancer-associated cachexia and weight loss [2,11]. The better profile of HA, including its anti-neoplastic action [1,5,31], may warrant examination of it for this application.

In this study, lifetime administration of HA did not significantly extend to lifespan of mice, in contrast to previous reports [15,16]. Positive observations have been ascribed to trapping of free radicals on the basis that antioxidants administered at high levels for a long time delayed certain ageing processes and reduced spontaneous tumor incidence [9,17,21,26]. Harman [18] suggested that the constant presence of HA, and possibly to a lesser extent other antioxidants, produces an environment detrimental to tumor cells. Our results revealed only a slight effect on lifespan, possibly due to the animal strain, since AKR, C3H and Swiss mice were only weakly responsive [17].

Another explanation for the absence of enhanced longevity in the present study may be the conditions under which the experiments were conducted. Kohn [23] showed that when the survival of experimental animals was optimal, antioxidants such as 2-mercaptoethylamine hydrochloride and butylated hydroxytoluene were without effect on the lifespan of C57B1 mice. When survival of controls was suboptimal, these agents caused lengthening of the lifespans, although in no case beyond that of mice surviving under optimal conditions. Our results support the conclusion that antioxidants do not affect those processes which determine maximum lifespans, although they may, nevertheless, counteract the action of some harmful factors in the environment, such as the oxidation of nutrients in the diet, which may shorten lifespan.

ACKNOWLEDGMENTS

The authors thank P. Radok for assistance with care and treatment of animals, Dr. Clare Mahan for statistical analysis, C. Meyer for preparation of histological material and T. Seppell for typing the manuscript.

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