Mechanisms of Ageing and Development, 27 (1984) 341–358
Elsevier Scientific Publishers Ireland Ltd.

EFFECT OF DIETARY 2-MERCAPTOETHANOL ON THE LIFE SPAN, IMMUNE SYSTEM, TUMOR INCIDENCE AND LIPID PEROXIDATION DAMAGE IN SPLEEN LYMPHOCYTES OF AGING BC3F$_1$ MICE

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(Received March 13th, 1984)
(Revision received July 4th, 1984)

SUMMARY

The age-related decline in immune function, which is thought to be responsible for the increased incidence with age of certain diseases, including cancer, has been attributed primarily to a loss of T-lymphocyte function. As free radical reactions may contribute to cellular deterioration and loss of cell function with age, we investigated the effect of adding an immunopotentiating antioxidant, 2-mercaptoethanol (2-ME), to the diet of BC3F$_1$ mice in a longitudinal study. For the study, young mice were divided into two groups, one of which received the 2-ME-supplemented diet. Approximately every 3 months for 2.5 years, mice from each group were sacrificed and the spleen lymphocytes assessed for immune function (proliferative response to concanavalin A, phytohemagglutinin, and lipopolysaccharide and the humoral response to sheep red blood cells). The accumulation of fluorescent products indicative of free radical damage was measured in the spleen lymphocytes and the cytochrome P-450 content and activity assessed in the liver. The effect of the 2-ME-supplemented diet on the mean and maximum life span and tumor incidence was also determined. The results showed that the animals fed the 2-ME diet had an increased mean and maximum life span and a postponed onset and decreased incidence of tumors. In general the T-cell-dependent immune responses were higher in the 2-ME-fed mice compared to the controls when the animals were young. No difference was observed between the two groups during mid-life. The responses declined in both groups during the latter half of the life span, but the responses of the 2-ME-fed animals declined to a lesser extent. The accumulation of fluorescent products of lipid peroxidation damage was also delayed in the lymphocytes of the 2-ME-fed mice. Cytochrome P-450 content and activity in the liver was not different in the two groups. The results suggest that the antioxidant activity of 2-ME delayed the accumulation of free radical damage in spleen lymphocytes, which resulted in a delay in the decline of immune function and was associated with the decreased tumor incidence and increased life span.

0047-6374/84/$03.00
Printed and Published in Ireland

Key words: 2-Mercaptoethanol; Antioxidant; Aging; Immune system; Free-radical damage

INTRODUCTION

Normal immune functions decline with advancing age in both animals and man, resulting in the increased incidence of some diseases and increased susceptibility to death [1–3]. The age-related decline in immune function is primarily due to a loss of thymus-derived lymphocyte (T-cell) function [3–5]. The underlying cause of this loss of T-cell function and the particular subclass(es) of T-cell(s) affected have not yet been totally defined. Age-related changes observed in cells of some other tissues have been attributed to free radicals producing lipid peroxidation damage and the accumulation of autofluorescent lipopigments within the cells [6,7]. In some animal studies, the addition of an antioxidant to the diet has resulted in a delay in the accumulation of the autofluorescent material and a prolonging of the life span of the animals [8–14], presumably because the antioxidant traps free radicals and reduces the amount of free radical damage in cells.

In 1972, Click *et al.* [15] reported that the antioxidant 2-mercaptoethanol (2-ME) enhanced the immune function of mouse lymphocytes when the compound was added to *in vitro* assays. Subsequently, it was demonstrated that 2-ME could enhance the age-suppressed responses of lymphocytes from old mice *in vitro* [16,17] and also *in vivo* [18,19]. In this study we added 2-ME to the diet of young mice and assessed in a longitudinal study the effect of the antioxidant on the life span, immune system, liver function and tumor incidence in the animals and determined the amount of lipid peroxidation damage in the spleen lymphocytes.

MATERIALS AND METHODS

Experimental design

The experimental design of the longitudinal study is shown in Fig. 1. Six hundred and sixty male $BC3F_1$/Cum mice (C57BL X C3H/Anf) were obtained from Cumberland View Farms, Clinton, TN, when 5 weeks old. When the mice were 16 weeks of age they were randomly divided into two groups; one group was fed a diet of Wayne Mouse Breeder Blox (Allied Mills, Inc., Chicago, IL) and the other group was fed the same diet with 0.25% (w/w) 2-ME added. Sixty animals from each group were set aside to determine the effect of the 2-ME-supplemented diet on the life span of the mice. These animals were kept until they died of natural causes. The remaining mice were used to determine the effect of 2-ME on the immune function, liver function, tumor incidence and accumulation of free radical damage in the spleen lymphocytes of the animals as they aged. Approximately every 3 months for the next 2.5 years at 21, 29, 42, 56, 70, 81, 95, 108, 121 and 134 weeks of age, 5 mice from each group were randomly selected, weighed, sacrificed and the spleens removed. The spleen cells from each individual mouse were assessed for their ability to respond to the mitogens, concanavalin A (Con A) and lipopoly-

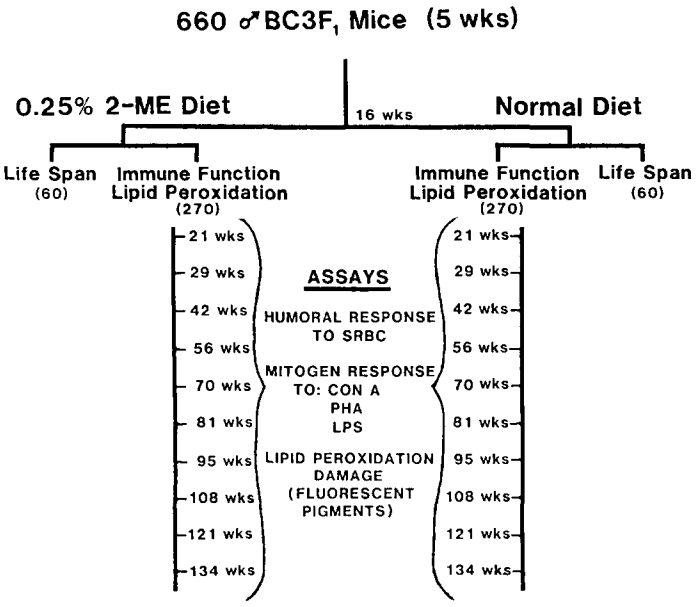

Fig. 1. Experimental design.

saccharide (LPS). In the latter part of the study, the response to phytohemagglutinin (PHA) was also assessed. The amount of lipid peroxidation damage in the spleen cells was determined by fluorescence analysis. To compare the humoral response of the two groups, at the same time periods, another 5 animals from each group were injected with sheep red blood cells (SRBC) and 7 days after injection, the mice were sacrificed, their spleens removed and the number of anti-SRBC antibody secreting cells determined. Liver function was assessed at 70 and 121 weeks by determining the activity of cytochrome P-450.

Preparation of diet

Because of the disagreeable odor of 2-ME, we were concerned that the experimental mice might eat less of the antioxidant-supplemented diet and that any results observed might inadvertently be the effect of caloric restriction. Consequently, before beginning the longitudinal study, several methods of incorporating the antioxidant into the animals' food or water were tried in order to determine the highest concentration of 2-ME the mice would tolerate while still eating the same amount of food as the control animals. Young mice were given either food or water containing various concentrations of 2-ME for a 4-week period and their weights monitored daily. At a concentration of 0.25% 2-ME or less, the experimental mice consumed the same amount of food as did the controls and maintained equal weights for the 1-month period. The animals refused to drink water with 2-ME in it and ate less of diets containing concentrations of 2-ME higher than 0.25%. To incorporate the 2-ME into the food, the mouse pellets were

weighed and then placed end to end in a single layer on shallow trays. The appropriate amount of 2-ME (0.25% of the weight of the pellets) was diluted in a volume of water previously determined necessary to moisten the pellets completely. One-half of the 2-ME solution was then sprayed onto the pellets in a fine mist. After drying, the pellets were turned over and the process repeated. The pellets were stored in air-tight containers. Fresh diet was prepared weekly.

Maintenance of aging mouse colony

The mice in the longitudinal study were maintained under barrier conditions according to guidlines* obtained from the National Institute on Aging. The mice were housed in shoe-box type cages (5 mice/cage) with polyester Econo-filter covers (Maryland Plastics, New York, NY). Cages, bedding, filter covers and water bottles were heat sterilized and the drinking water was sterilized by filtration though Millipore filters. Animal caretakers and investigators entering the room were required to wear gowns, plastic shoe covers, masks and gloves. Every 6 months during the study, blood samples from randomly selected animals were sent to Microbiological Associates Virus Diagnostic Laboratory, Bethesda, MD, for virus testing. At no time during the study did the serum samples show a positive test for any murine virus, such as Sendai virus or ectromelia virus, which would have affected the results of the study.

Chemicals

2-ME was obtained from Eastman Organic Chemicals, Rochester, NY. Con A was purchased from Sigma Chemical Co., St. Louis, MO, PHA (M form) from Grand Island Biological Co., Grand Island, NY, and LPS (*E. coli* 055:B5) from Difco Laboratories, Detroit, MI. The optimum concentration of each mitogen lot was determined experimentally using spleen lymphocytes from both young and old mice. For all three mitogens, the concentration required to produce maximal stimulation in young lymphocytes also produced maximal stimulation in the old lymphocytes. Tritiated thymidine [*methyl*-^{3}H] was obtained from New England Nuclear, Boston, MA.

Mitogen assays

The proliferative response of the spleen lymphocytes to the T-cell mitogens, Con A and PHA, and to the B-cell mitogens, LPS, was determined by culturing the lymphocytes with the optimum concentrations of the mitogens and subsequently assessing the amount of proliferation by measuring the amount of [^{3}H] thymidine incorporated into the DNA of the dividing cells. The procedures for preparing the spleen lymphocyte suspensions, cell cultures and for harvesting the cells have been reported elsewhere [17]. Each animal was assayed individually with ten replicate cultures.

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*Personal communication, Don C. Gibson, DVM, MPH, National Institute on Aging.

Humoral immune response

The humoral immune response of the mice was assessed by determining the ability of the animals to produce antibodies to SRBC. Five animals from each group were randomly selected and injected intraperitoneally with 0.1 ml of a 20% suspension of SRBC. The SRBC were obtained from Grand Island Biological Diagnostic Laboratories. A fresh suspension of SRBC from the same donor sheep was obtained for each assay time over the 2.5-year period. Seven days after injection of the antigen, the mice were sacrificed and a spleen cell suspension was prepared as previously described [17]. The number of spleen cells secreting anti-SRBC antibody was then determined using a hemolytic plaque assay. The slide method (gel) modification [20] of the procedure originally developed by Jerne *et al.* [21] was used. The results were calculated both as the number of antibody-forming cells per spleen and as the number of antibody-forming cells per 10^6 spleen cells.

Liver function

Mouse liver microsomes were prepared and assays for microsomal aminopyrine and aniline metabolism were conducted as previously described [22], except that 50 μM EDTA was added to reaction mixtures with aminopyrine as substrate. Total cytochrome P-450 concentrations were determined from the dithionite-reduced CO-difference spectra using the extinction coefficient of 91 mM^{-1} cm^{-1} as described by Omura and Sato [23]. Microsomal protein concentrations were determined by the method of Lowry *et al.* [24].

Measurement of fluorescent lipid peroxidation products

The amount of free radical damage occurring in the spleen lymphocytes was assessed by measuring the fluorescent lipid peroxidation products as described by Fletcher *et al.* [25]. Equal numbers of spleen lymphocytes (10×10^6) from the 2-ME-fed and control mice were taken from the cell suspensions prepared for mitogen assay. The cells were centrifuged, rinsed and then lysed by freezing and thawing. The cell lysates were centrifuged and then extracted with 3 ml of chloroform–methanol (2:1, v:v). All procedures were carried out under incandescent light. Interfering fluorescent flavin compounds were removed by a water wash and interfering retinol was removed by exposing the chloroform-rich extract to high-intensity ultraviolet light for 30 sec. The excitation (360 nm) and emission (430 nm) fluorescence spectra of the extracts were measured with an Aminco-Bowman spectrophotofluorometer. Quinine sulfate at a concentration of 1 μg/ml of 0.1 N H_2SO_4 was used as a standard for fluorescence intensity and wavelength calibration.

Analysis of data

Lymphocyte responses to mitogens and antigens were log-transformed and analyzed by *t*-test, using the Alpha 325 Univariate Statistics Pak and Significance Pak for a Litton Monroe Alpha 325 Scientist programable calculator.

RESULTS

Effect of 2-ME on life span

The survival curves of the $BC3F_1$ mice fed either the control or 2-ME-supplemented diet are shown in Fig. 2. The mean survival time for the mice fed the control diet was 114.6 ± 3.2 weeks (± standard error) whereas the mice fed the 2-ME diet had a significantly greater ($p < 0.005$) mean survival time of 129.1 ± 3.0 weeks, a 13.2% increase in mean life span. Maximum survival for the control animals was 150 weeks compared to 168 weeks for the 2-ME-fed animals, a 12.0% increase in maximum survival time. The mean survival time for the longest lived 10% of each group ($N = 6$) was 144 ± 1.5 weeks for the control mice and 159 ± 1.9 weeks for the 2-ME-fed mice. This represents a significant increase ($p < 0.001$) in the mean survival of the longest lived 10% of the population.

The Gompertz function [26], or relationship of the death rate to age for the two groups after 84 weeks of age, is plotted in Fig. 3. The plot indicates that the Gompertz function for the 2-ME group is displaced downward from the control group but the slope of the line for the 2-ME group (0.055012) is not significantly different from the slope of the line for the control group (0.058744). Thus 2-ME did not alter the mortality rate of the mice but delayed the onset of exponential death with age.

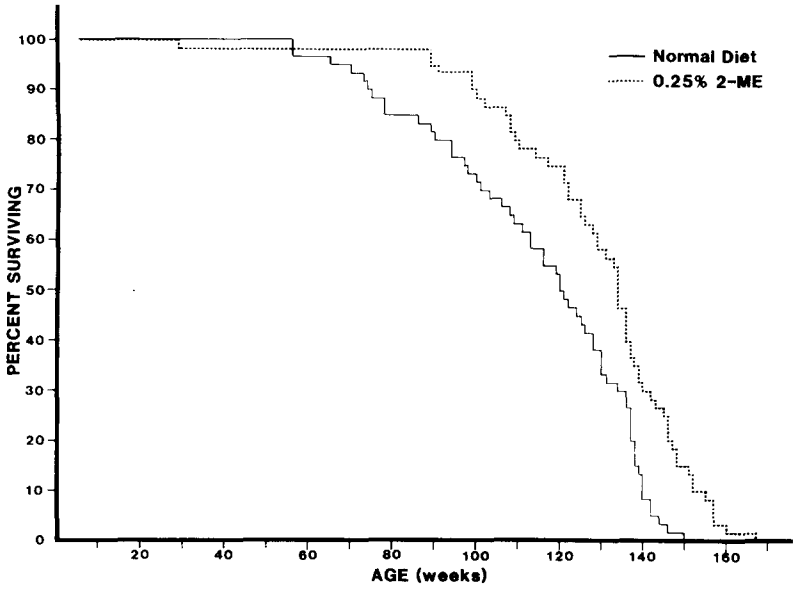

Fig. 2. Effect of dietary 2-ME on the life span of $BC3F_1$ mice. Two groups of 60 mice each were randomly selected from a total of 660 animals. When the mice were 16 weeks of age, one group was changed to a diet containing 0.25% (w/w) 2-ME. The animals were allowed to die of natural causes and the number of deaths were recorded daily. Both the mean and the maximum life span of the two groups were significantly different.

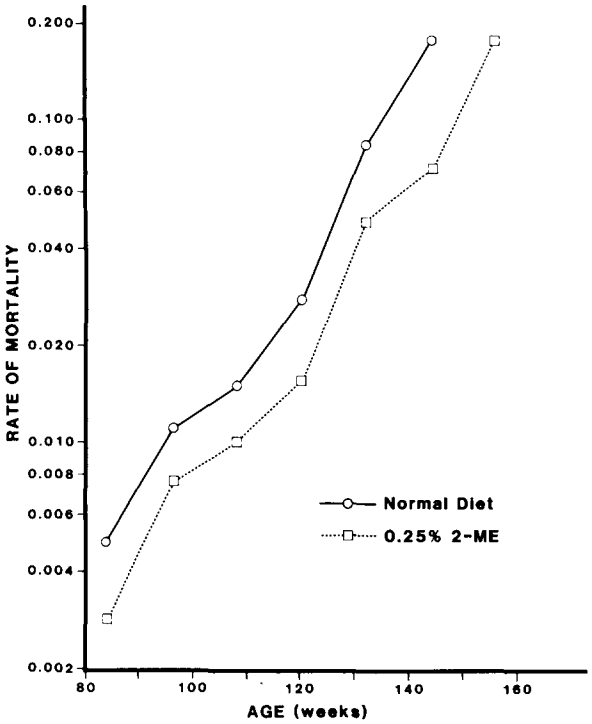

Fig. 3. Comparison of Gompertz curves for the control and 2-ME-fed groups. Age-specific rates of mortality after 84 weeks of age were plotted on a logarithmic scale *versus* age. Mortality rates, w_t, were calculated as

$$w_t = \frac{1}{2h} \log_e \frac{N(t-h)}{N(t+h)}$$

where $2h$ is the time interval (12 weeks) and N(t) is the number of animals living at time t.

Body weight, spleen weight and food consumption

The animals, randomly selected for the assays during the longitudinal study, were weighed immediately before sacrifice to monitor their weight. The results are shown in Table I. For unexplained reasons, the control animals selected for assay at 56 weeks weighed significantly more than the 2-ME-fed group. At all other assay points, there was no significant difference in the weights of the two groups. The amount of food consumed by the two groups was periodically monitored by measuring the weight of control or 2-ME-supplemented pellets consumed by representative cages of the animals in the life span study. For the first half of the life span there were no differences in the amount of food consumed by the test animals in the two groups. However, after about 80 weeks of age until 115 weeks of age the 2-ME-fed animals consumed approximately 10–20% more of their diet than did the control animals, although, as previously indicated, the body weights were not significantly different during this time. The spleen

TABLE I

BODY WEIGHTS AND SPLEEN WEIGHTS OF CONTROL AND 2-ME-FED MICE AT DIFFERENT AGES

The body and spleen weights of the animals randomly selected for sacrifice were measured at each assay time. Values are the average of at least 10 animals/group ± S.E.M.

Age (weeks)	*Body weights (g) ± S.E.M.*		*Spleen weights (mg) ± S.E.M.*	
	Control	*2-ME-fed*	*Control*	*2-ME-fed*
21	41.5 ± 1.5	41.5 ± 1.3		
29	46.4 ± 1.0	46.4 ± 1.0		
42	43.4 ± 1.4	41.4 ± 1.1	131.2 ± 8.9	129.6 ± 9.3
56	56.7 ± 0.6	45.1 ± 0.7[a]	162.8 ± 9.0	138.6 ± 6.5[a]
70	52.1 ± 0.6	53.8 ± 0.8	164.4 ± 9.6	146.8 ± 8.9
81	52.6 ± 1.9	53.8 ± 0.9	187.3 ± 15.0	152.7 ± 11.0
95	37.8 ± 3.2	42.7 ± 3.0	131.0 ± 9.5	207.6 ± 30.9[a]
108	41.8 ± 3.0	41.6 ± 1.5	186.0 ± 31.9	154.5 ± 16.5
121	45.5 ± 2.2	42.6 ± 1.3	184.4 ± 16.8	182.4 ± 10.1
134	37.8 ± 2.1	41.5 ± 1.4	158.0 ± 16.8	233.2 ± 27.3[a]

[a]Values for 2-ME-fed animals significantly different from controls ($P < 0.05$). The body weights of the control animals recorded at 56 weeks seem unusually high in our experience with this strain of mice and it is possible that the balance scale was misread or incorrectly tared when the animals were weighed.

weights for the two groups were significantly different at 56, 95, and 134 weeks of age ($p < 0.005$), but no consistent pattern of difference was observed, *i.e.* at 56 weeks, control > 2-ME; at 95 weeks, control < 2-ME; and at 134 weeks, control < 2-ME.

Effect of 2-ME on lymphocyte proliferative response to mitogens

A comparison of the proliferative response of spleen lymphocytes from the 2-ME-fed and control mice to the T-cell mitogen, Con A, during the longitudinal study is shown in Fig. 4. The 2-ME-fed mice demonstrated a significantly higher response early in the study. During mid-life no difference between the two groups was observed. As the animals grew older the normal response declined at a faster rate such that, in the later part of the study, the 2-ME-fed mice again showed a higher response than the controls. The response of the 2-ME-fed mice was significantly higher than the controls during the last 6 months of the study (at 121 and 134 weeks of age). Beginning at 70 weeks of age, the response of the lymphocytes to a second T-cell mitogen, PHA, was measured (Fig. 5). Similar to the response to Con A, the PHA response of the 2-ME-fed animals declined at a slower rate than the controls, leading to an enhanced response by the 2-ME-fed animals over the controls, which was significantly greater during the last 6 months of the study. The response of the two groups to the B-cell mitogen, LPS, is shown in Fig. 6. The 2-ME-fed mice had a higher response to this mitogen at only two time points, 29 weeks and 95 weeks, and the magnitude of the greatest enhanced response (at 95 weeks)

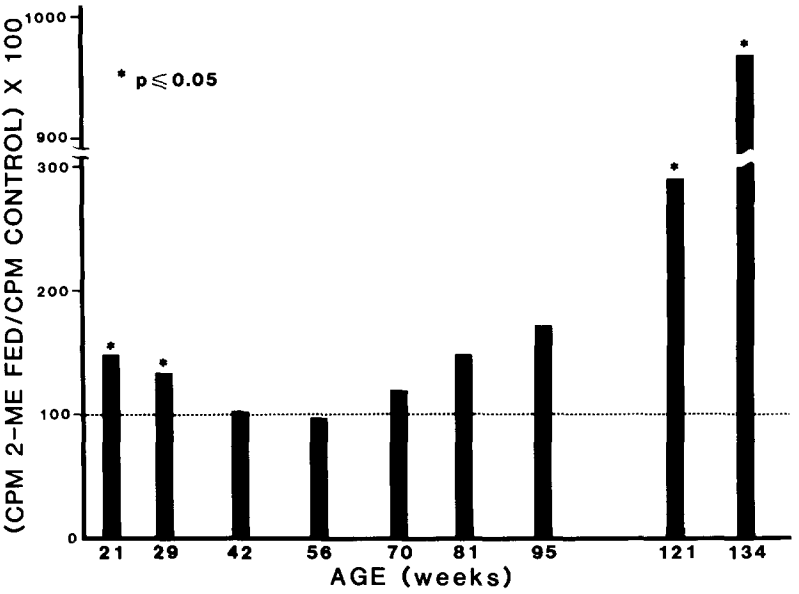

Fig. 4. Response of spleen lymphocytes from the 2-ME-fed and control mice to the T-cell mitogen Con A. The proliferative response was measured as the cpm [^{3}H]thymidine incorporated into DNA of proliferating cells. At the indicated ages, 5 mice from each group were randomly selected and tested individually (10 cultures/mouse). The average response of the 5 2-ME-fed mice (solid bars) is indicated as a percentage of the average control response (dotted line) set at 100% (*i.e.* when the solid bars are above the dotted line, the 2-ME-fed animals demonstrated a greater response than the control animals). The highest response for both groups (controls 62 767 cpm/culture, and 2-ME-fed 63 242 cpm/culture) was observed at 42 weeks after which the response in both groups declined, but to a lesser degree in the 2-ME-fed mice (*e.g.* at 134 weeks: controls 3479 cpm/culture, and 2-ME-fed 33 395 cpm/culture).

was much less than that observed with the T-cell mitogens. No enhancement over the controls was observed during the latter part of the longitudinal study.

We were interested in whether the 2-ME diet was maximally stimulating the cells or if they could be further stimulated by adding additional 2-ME to the *in vitro* cultures. Consequently, at one time point, 29 weeks, when the 2-ME-fed mice demonstrated an enhanced response over the controls, we added 2-ME (5×10^{-5} M) to replicate lymphocyte cultures. The results (Table II) indicate that further stimulation of the lymphocytes was possible by adding 2-ME to the cultures.

Effect of 2-ME on the humoral immune response

The ability of the control and 2-ME-fed mice to produce antibodies to a T-cell-dependent antigen (SRBC) throughout the study is shown in Fig. 7. The response of the 2-ME-fed animals was significantly higher than the control animals at 56 weeks of age and also in the latter part of the life span at 95, 108 and 121 weeks of age. At the very last assessment (134 weeks), the humoral response of the 2-ME-fed mice was higher than the controls, but the increase was not significant ($P > 0.05$).

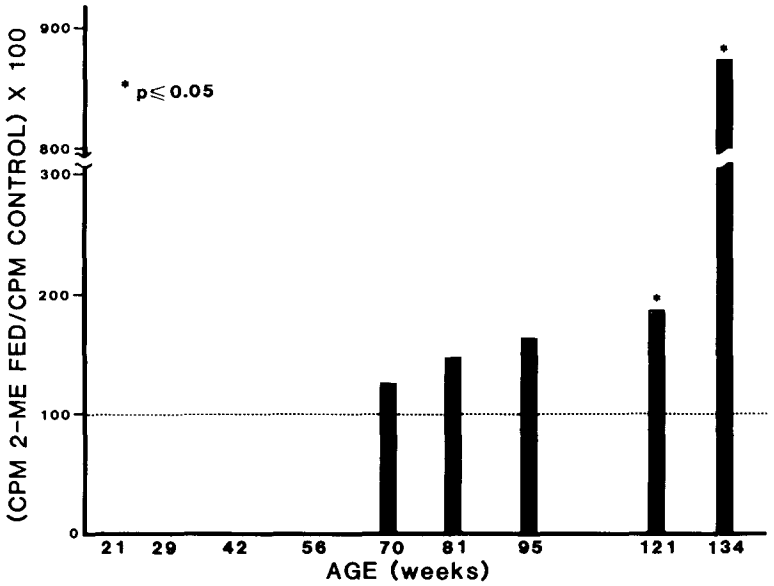

Fig. 5. Response of spleen lymphocytes from the 2-ME-fed and control mice to the T-cell mitogen PHA. The proliferative response was measured as the cpm [^{3}H]thymidine incorporated into DNA of proliferating cells. At the indicated ages, during the latter part of the longitudinal study, 5 mice from each group were randomly selected and tested individually (10 cultures/mouse). The average response of the 5 2-ME-fed mice (solid bars) is indicated as a percentage of the average control response (dotted line) set at 100%.

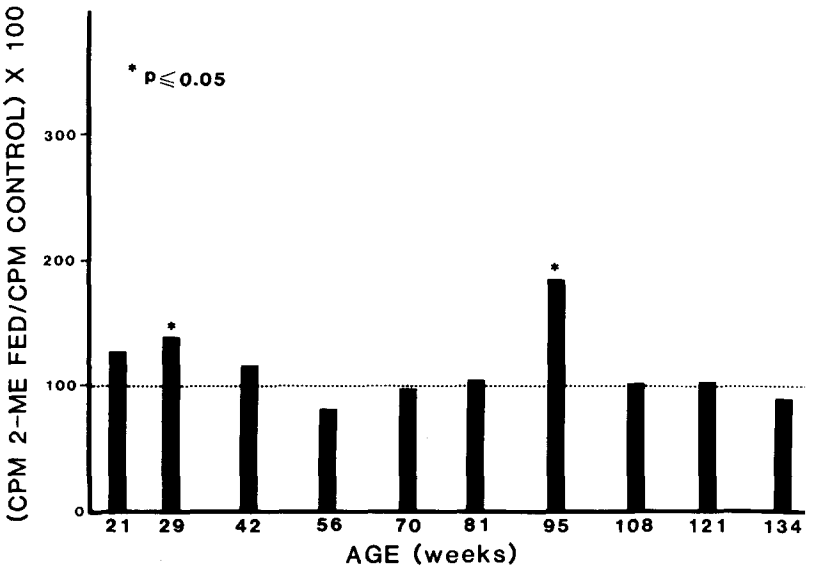

Fig. 6. Response of spleen lymphocytes from the 2-ME-fed and control mice to the B-cell mitogen LPS. The proliferative response was measured as the cpm [^{3}H]thymidine incorporated into DNA of proliferating cells. At the indicated ages, 5 mice from each group were randomly selected and tested individually (10 cultures/mouse). The average response of the 5 2-ME-fed mice (solid bars) is indicated as a percentage of the average control response (dotted line) set at 100%. The highest response for both groups was observed at 42 weeks (control 13 834 cpm/culture; 2-ME-fed, 16 094 cpm/culture).

TABLE II

EFFECT OF ADDING 2-ME TO *IN VITRO* CULTURES OF LYMPHOCYTES FROM 2-ME-FED AND CONTROL MICE

Values are the mean cpm/culture ± S.E.M. of lymphocyte cultures from 5 control and 5 2-ME-fed mice (10 cultures/mouse). 2-ME was added at a concentration of 5×10^{-5} M. The mice were 29 weeks of age and the experimental animals had received the 2-ME-supplemented diet for 13 weeks.

Additions to culture	*cpm/culture ± S.E.M.*		*2-ME-fed/ control*	*With 2-ME/without 2-ME*	
	Control	*2-ME-fed*		*Control*	*2-ME-fed*
None	542 ± 15	538 ± 12	0.99		
Con A	26,641 ± 1,335	35,418 ± 3,986	1.33	1.78	1.53
Con A + 2-ME	47,536 ± 1,111[a]	54,273 ± 1,674[a]	1.14		
LPS	9,099 ± 728	12,762 ± 919	1.40	3.11	2.45
LPS + 2-ME	28,350 ± 2,120[a]	31,288 ± 1,550[a]	1.10		

[a]Cultures with 2-ME added *in vitro* had significantly higher cpm ($P < 0.005$) for both control and 2-ME-fed mice.

Liver function

In order to determine if 2-ME feeding affected hepatic microsomal (endoplasmic reticulum) drug metabolism function, the metabolism of model type I and type II substrates, aminopyrine and aniline, respectively, [27] was examined with microsomes from control and 2-ME-fed mice at 70 weeks. The data shown in Table III indicate that

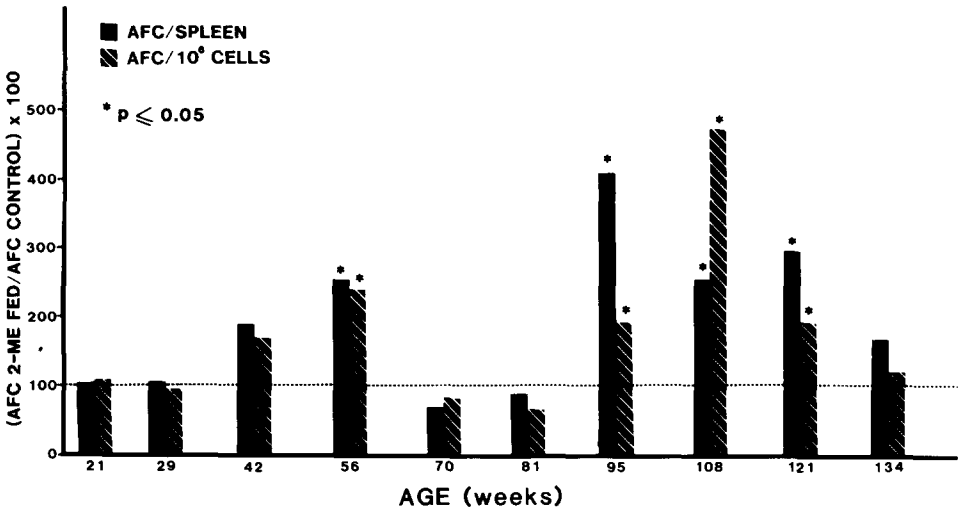

Fig. 7. Effect of 2-ME on the humoral response to SRBC. The response was measured as the number of antibody-forming cells (AFC) per spleen and as per 10^6 spleen cells in 2-ME-fed or control mice, 7 days after injection with SRBC. The average response of the 5 2-ME-fed mice (solid and striped bars) is indicated as a percentage of the average control response (dotted line) set at 100%. The highest response for both groups was observed at 56 weeks (control, 36 108 AFC/spleen; 2-ME-fed, 91 728 AFC/spleen).

TABLE III

LIVER WEIGHTS AND HEPATIC DRUG METABOLISM FUNCTION OF CONTROL AND 2-ME-FED MICE AT 70 WEEKS

The values shown are the mean ± S.E.M. (number of livers). The differences between the control and 2-ME fed groups were not significant ($P > 0.05$) for liver weight, aminopyrine metabolism, aniline metabolism and cytochrome P-450 content.

	Liver weight (g)	*Aminopyrine metabolism (formaldehyde produced, nmol/min/mg protein)*	*Aniline metabolism (p-aminophenol produced, nmol/min/mg protein)*	*Cytochrome P-450 (specific content, nmol/mg protein)*
Control	2.88 ± 0.17 (5)	5.42 ± 0.05 (4)	1.35 ± 0.09 (4)	0.67 ± 0.03 (4)
2-ME Fed	2.97 ± 0.28 (5)	6.39 ± 0.38 (5)	1.58 ± 0.10 (5)	0.77 ± 0.09 (5)

2-ME feeding had no significant effect on these enzyme-catalyzed reactions. The data also indicate that neither the liver weights nor the specific content of cytochrome P-450, the terminal oxidase of the membrane-located enzymatic system that catalyzes these reactions, were significantly affected by the 2-ME diet. At 121 weeks of age, only 2 of the 5 animals sacrificed from either the control or 2-ME-fed group were judged free of liver pathologies and suitable for assay. However, even at this age these normal livers of both groups maintained a cytochrome P-450 specific content (0.63 in control and 0.72 in 2-ME-fed, each an average of two livers) similar to that seen at the earlier age (Table III). Liver weights at this age, however, were decreased (1.87 g in control and 1.67 g in 2-ME-fed) compared to liver weights at 70 weeks (Table III).

Effect of dietary 2-ME on lipid peroxidation damage in spleen lymphocytes

Damage to cell membrane structures by lipid peroxidation from free radical reactions appears to be an important deteriorative mechanism of cellular aging, and the amount of damage can be measured by determining the age-related accumulation of soluble fluorescent products of lipid peroxidation in the cells [25]. Figure 8 shows the effect of dietary 2-ME on the accumulation of soluble fluorescent products with age in the spleen lymphocytes. The spleen lymphocytes from the 2-ME-fed mice had significantly less soluble autofluorescence than did the control mice at 95, 108, 121 and 134 weeks of age ($p < 0.05$).

Tumor incidence

The development of tumors in the animals was assessed by gross examination of the ten or more animals sacrificed from each group at the 3-month intervals. Hepatomas were the most frequent tumors observed, followed by lymphomas and renal tumors. Figure 9 shows the percentage of animals with tumors at each assay point. The first tumors appeared later (56 weeks) and the total incidence of tumors was less (29%) in the 2-ME-fed group compared to the controls (42 weeks, 50%).

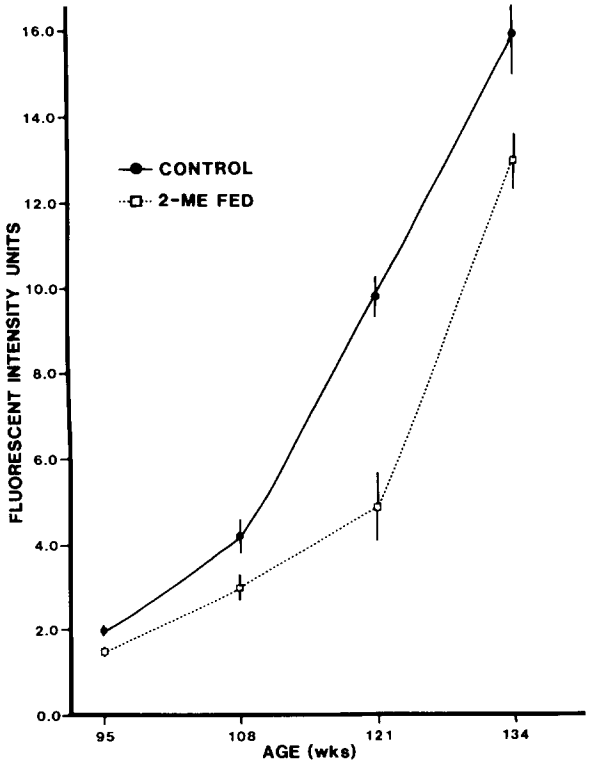

Fig. 8. Effect of dietary 2-ME on the accumulation of fluorescent products in spleen lymphocytes. Aliquots containing equal numbers (10×10^6) of nucleated spleen cells (after removal of red blood cells with ammonium chloride) were extracted with chloroform–methanol and analyzed for lipid peroxidation damage on a spectrophotofluorometer. The fluorescence intensity units in the figure were derived by multiplying the meter multiplier value by the percent transmission. The values are the averages of 10 individual mice from each group ± S.E.M.

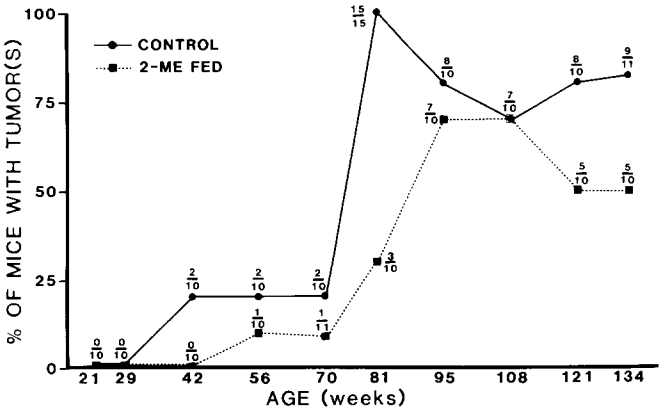

Fig. 9. Incidence of tumors in the 2-ME-fed and control mice. Each of the 10 or more mice from the two groups selected for assessment of mitogen (Figs. 4–6) and antibody (Fig. 7) responses were examined immediately after sacrifice for the presence of tumors. Animals having tumors involving the spleen were not used for spleen cell assays, consequently more than 10 animals were needed for some age points. Values are (the number of animals with tumors/the total number of animals sacrificed) × 100.

DISCUSSION

The objective of our research is to develop a method to prevent or delay the onset of chronic diseases of aging in humans and to decrease the proportion of the life span spent suffering from the effects of chronic disease, thereby decreasing the high costs of medical care for our increasing numbers of elderly. Such a strategy will be successful only if the maximum life span of man does not increase [28,29]. For this reason, we were particularly interested in antioxidants because of previous studies by others which showed an increase in mean life span without an increase in maximum life span [11,13,14]. Some investigators, however, have reported an increase of maximum life span with antioxidants [9,10,14]. This study confirms the work of others that antioxidants, when incorporated into the diet, will extend the mean life span of mice. In our study an increase in the maximum life span was also observed. In addition, our study correlates this extension of life span with a delay in the decline of immune function and a decreased incidence of tumors. It is not clear why some antioxidant studies show a maximum life span increase and others do not. Although the observed increase in the maximum life span in our study was not necessarily a desirable result, it may be possible to eliminate an extension in maximum life span by removing the antioxidant from the diet during later life.

Walford's group [30–32] has demonstrated that the decline in immune function can be delayed and life span extended by caloric restriction. It has been suggested that in previous studies of antioxidants, the experimental animals may not have eaten as much of the antioxidant-supplemented diet as did the control animals and the reported effects may have inadvertently been due to caloric restriction rather than the antioxidant [9]. In the present study, the concentration of 2-ME added to the diet was chosen only after determining that the animals receiving the experimental diet consumed as much as did the control animals. Total body weights of the two groups were not different except at one time point, 56 weeks (Table I). During the third quarter of the life span (80–115 weeks), the 2-ME-fed animals actually consumed more (10–20%) of their diet than did the controls, although the body weights were not significantly different. During the later part of the study, the 2-ME-fed mice were observed to be more active than the control animals and generally presented a better physical appearance (less hair loss, healthier appearing coat). It is possible that during this time the 2-ME-fed mice utilized more calories than did the less active controls. In diet restriction studies, the very young diet-restricted mice have lower than normal immune responses and a higher incidence of early deaths than the control mice [30]. In contrast, in the present study the very young 2-ME-fed mice had higher than normal responses to Con A and LPS (Figs. 4 and 6) and early deaths were not a problem.

The data shown in Table III indicate that inclusion of 2-ME in the diet under the conditions used had no significant stimulatory or inhibitory effect on liver size, cytochrome P-450 specific content or cytochrome P-450 mediated hepatic function. Other studies have indicated that antioxidants result in stimulation [10], inhibition [33] or

selective stimulation and inhibition [34] of hepatic microsomal mixed function oxidase activity. Also, others have suggested that stimulation of immune function may alter hepatic microsomal enzymes [35]. However, the data shown in Table III indicate that this system was apparently not significantly perturbed by the 2-ME feeding regime used.

The exact mechanism by which dietary 2-ME produced the observed effects on the immune function of the 2-ME-fed mice is not known but several modes of action may be involved. The delay in the accumulation of lipid peroxidation damage with age in the spleen lymphocytes from the 2-ME-fed mice (Fig. 8) correlated with the slower decline of T-cell function [proliferative response to Con A (Fig. 4) and PHA (Fig. 5) and the slower decline of the humoral response to the T-cell-dependent antigen SRBC (Fig. 7)]. This suggests that the accumulation of lipid peroxidation damage in T-lymphocytes may be in part responsible for the loss of T-cell function with age and that the dietary 2-ME acted as a free radical inhibitor or enhanced other free radical scavenging capacity. This view is strengthened by our further studies showing that T-cells accumulate more of the fluorescent lipopigments indicative of lipid peroxidation damage with age than do B-cells and that young T-cells are more susceptible to free radical damage than young B-cells [36]. However, it is also apparent that the dietary 2-ME influenced the lymphocyte responses by some other mechanism(s) because the responses of very young mice were also enhanced and the long-term accumulation of lipid peroxidation damage should not be a problem early in life. The ability of 2-ME to enhance the responses of old lymphocytes both *in vivo* [18,19] and *in vitro* [16,17] without prior exposure or long-term exposure to the antioxidant also suggests a second mechanism in addition to long-term prevention of free radical damage.

Studies of the action of 2-ME in enhancing lymphocyte function *in vitro* have resulted in a number of proposed mechanisms of action including activating a component of fetal calf serum which is able to substitute functionally for macrophages [37], promoting the production of T-cell growth factor (interleukin 2) [38], forming a mixed disulfide with cysteine (2-ME-cysteine) and thus facilitating the uptake of cysteine [39], and enhancing the availability of reduced glutathione thought to be necessary as a free radical scavenger to protect lymphocytes and macrophages from oxygen-derived radicals arising from the high pO_2 in tissue culture conditions and from the oxygen-derived radicals produced by polymorphonuclear neutrophils and macrophages [40,41]. The latter study suggests that natural protective mechanisms in the cell may not be sufficient to prevent the destructive effects of lipid peroxidation even in lymphocytes from young mice and that additional short-term free radical scavenging capacity is beneficial at least *in vitro*. This is also supported by our study showing that the addition of 2-ME to *in vitro* cultures further stimulated lymphocytes from 2-ME-fed mice to proliferate (Table II). Whether these mechanisms are responsible for the enhanced activity seen in the lymphocytes from the young 2-ME-fed mice remains to be determined.

There are varying opinions about how effective the immune system is in destroying malignant cells *in situ* and preventing the development of cancer. However, increasing evidence indicates the age-related loss of immune function is at least in part responsible

for the age-related increase in the incidence of cancer. Old mice with suppressed immune function are more susceptible to transplanted tumor cells [42] and to carcinogens [43]. Treatments known to delay the age-related decline of immune function or improve the age-depressed immune system decrease the incidence of tumors and increase longevity [44–47], although in diet restriction studies tumor frequency varies with the portion of the life span during which restriction is imposed [31]. Dietary antioxidants decrease the incidence of spontaneous [48] and carcinogen-induced tumors [10,49]. In the studies of carcinogen-treated mice, it has been suggested that antioxidants inhibit the development and/or growth of tumors by influencing the metabolism of the carcinogen (increasing the synthesis of cytochrome P-450), and/or inhibiting the growth of the tumor cells [10,49]. (It is also possible that the antioxidants decrease the incidence of tumors by preventing free radical damage to lymphocytes and delaying the decline of immune function.) In this study, the mice were maintained in barrier conditions and not purposely exposed to any carcinogens. Cytochrome P-450 content and activity were not different between the control and 2-ME-fed mice at the two times measured (Table III), yet the 2-ME-fed mice developed fewer spontaneous tumors. The appearance of the first tumor was delayed for a time period similar to the observed delay in the decline of immune function, the delay in the accumulation of lipid peroxidation damage in spleen lymphocytes and the increase in mean and maximum life span.

CONCLUSIONS

The decreased incidence of tumors and increased mean and maximum life span observed in the $BC3F_1$ mice fed a diet supplemented with 2-ME may be due to the antioxidant activity of 2-ME retarding the accumulation of free-radical damage in lymphocytes, and delaying the age-related loss of immune function.

ACKNOWLEDGEMENTS

The authors thank Laura A. Gaul, Joy A. Yates, John Jackson and Kimberly A. Hanson for excellent technical assistance. This research was supported by the National Institute of Aging, Grant No. AG00904.

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