Obesity, longevity, quality of life
Alteration by dietary 2-mercaptoethanol

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Introduction

One important means to define metabolic and physiological processes is to identify postulated intermediary molecules and suspected enzymes/proteins and then purify and characterize them. In many cases, retention of activity is dependent upon the presence of a strong sulfhydryl reagent, such as 2-mercaptoethanol (2-Me) or dithiothreitol, to maintain essential thiol/disulfide bonds. Because such critical structural conformations are essential for function, investigations were undertaken to determine what effect sulfhydryl molecules might impart on immune cell reactivities in tissue culture under a non-physiological, oxygen environment. We first tested, if the then, Mishell/Dutton protocol for in vitro primary murine antibody synthesis at physiological levels of oxygen, could be replaced by addition of 2-Me to cultures that were incubated in air. We found that 2-Me addition eliminated shaking of culture vessels, daily feeding, and low levels of oxygen, while maintaining equivalent cell viability and function. This finding led to a series of investigations that established sulfhydryl dependent, culture protocols for primary serum-free murine mixed lymphocyte reactions (MLR), isologous/homologous-serum supplemented murine MLR, and swine and bovine whole blood immune functions [Click, unpublished].

Previous investigations demonstrated that optimization of murine immunological reactivity in tissue culture required a sulfhydryl compound; the most effective being 2-mercaptoethanol (2-Me). Since these reports, 2-Me was found beneficial for both growth/function of other cell-types in vitro, including those of other species, and when fed orally, it impeded and/or reversed some in situ physiological changes associated with aging. More recently, thiol-containing compounds possessing oxidation-reduction potentials weaker than 2-Me were found to impart beneficial effects for many other, including human, diseases. Based on these effects, the research herein addressed the question: What consequences might dietary 2-Me impart on health and disease of mice other than those associated with aging? The main parameters monitored over the lifetime of individual animals exposed to dietary 10^-3 M 2-Me in their drinking water were: quality of life (obesity and development of recumbent, emaciated and/or cachectic health, longevity, and appearance of tumors. Instead of anticipated toxic attributes, the following unique benefits were found: mean survival of a moderately-lived strain (A/J) was increased 40.8%, high-fat-diet obesity was curtailed in C57BL/10 mice, and a goal of aging intervention protocols, namely preventing loss of quality of life during aging (recumbent, emaciated and/or cachectic) was achieved. Various mechanisms are discussed as they pertain to these findings.
Results

Longevity. The single investigation of 2-Me’s effect on longevity found that the mean survival of long-lived (C57BL x C3H)F1 mice fed the high fat, Wayne breeder diet, was statistically extended from 802 days to 904 days (12.7%) by mixing 2-Me with the feed at a concentration of 0.25%. To test whether this longevity increase was dependent on strain of mice and/or diet, groups of C57BL/10 mice (n = 14) started on 2-Me treatment at 70 days of age, and non-treated control mice (n = 14) were fed one of four different commercial diets that differed primarily in the percentage of fat (referred to as 4P%, 6T%, 10T% and 10W%). The latter diet is the same as that used in the longevity investigations reported for (C57BL x C3H)F1 mice. As shown in Figure 1, although no statistically significant differences were found in mean survival of treated vs. non-treated mice on any given diet (see legend), median survival of treated mice on the latter three diets increased from 957 to 981 (2.5%), 708 to 872 (23%) and 834 to 911 (9.2%) days, respectively. Treated mice on the 10%T diet had a reduced median time (9.9%) of 815 from 905 days. Even though there were some significant differences in survival of animals on different diets, these comparisons will not be discussed since they are not the focus of this report.

Health parameters. In addition to monitoring longevity, body-mass, ‘overall well-being’, and cholesterol levels were also determined. At death, those not excessively autolyzed were necropsied. Shown in Figure 2 are changes in the average body mass ± SD of treated and nontreated mice (n = 14 per treatment) on the four different diets. For each diet, the average maximum weight of nontreated animals was significantly (p < 0.01) greater than that of animals treated with 2-Me. Changes for individual mice on 10%T and 4%P diets that survived at least 810 days are shown in Figure 3. Nontreated mice on the two high-fat diets at 370 days were considered obese based on extensive documentation of obesity at similar weights in C57BL males. In contrast, body mass of 2-Me treated animals on these two high-fat diets was much more stable once maturity was reached and the animals never became obese. Nontreated animals fed the low-fat diets also did not become obese and their weight fluctuated less than that of those on the high-fat diets. Body mass of those treated with 2-Me and fed the two low-fat diets was very stable; indeed, weight of most actually increased with age (Fig. 3). Body mass for both nontreated, as expected, and for treated animals was directly associated with the percentage of fat in the diet. Interestingly, nontreated mice on the two high fat diets, not only gained, but also eventually lost, weight

release, and (g) prevented malondialdehyde formation. This latter chemical is implicated in irreversible cross-linking of proteins and DNA, which is formed along with lipofuscin during autooxidation of polyunsaturated fatty acids. Because these in situ findings were made with different strains of mice, different modes of delivery (injection, mixed with food or water), different doses (ranged from 0.1 to 15 ug/gm body weight), different ages at which treatment was initiated, and different diets, the experiments herein were undertaken to further test 2-Me for other benefits. In this first of a series, the focus was on changes in longevity, obesity and overall age-dependent well-being. Since the results were unpredictable, no animals were sacrificed to assess molecular or biochemical parameters; all died of natural causes.
much faster than those on the low fat diets. Most animals in the four nontreated groups died recumbent, emaciated and cachectic at weights considerably lower (not shown because of limited numbers of animals) than shown at 810 days. In contrast, treated animals on the two high-fat diets lost less weight with age, whereas those on the low-fat diets either maintained or slightly gained weight up until their death; i.e., treated animals did not die recumbent, emaciated and/or cachectic; indeed, they appeared to have more vigor.

To assess whether differences in weight gain/loss were a consequence of greater/lesser food/water intake, and/or related to calories associated with fat content of the diets, the amount of food consumed was determined at two different ages; that prior to and that at maximum weight. Consumption of water was determined at only a single age. As shown in Table 1, the average consumption of water containing 2-Me, per mouse, was greater than that of normal water for all four diets. This was most dramatic for animals on 6%T and 10%W diets. The average 2-Me consumed/day/gm body weight ranged from 8.5 ug/m (10%T diet) to 15.7 ug/m (10%W diet). In contrast, average feed intake of a given diet by treated and nontreated animals was similar at a given age. The average/mouse of 6%T and 10%T feed consumed at 370 days was less than at 205 days, whereas that for the other two feeds was similar at both times. The only other difference was that less 10%W food was consumed at 205 and 370 days and more 4%P food was consumed at 370 days relative to other diets.

Serum cholesterol levels of nonfasted B10 animals on a given treatment and diet at 395 and 760 days of age were similar and were therefore combined for analysis. The means and standard errors are shown in Figure 4 for each diet. For 3 of the 4 diets, cholesterol was lower in treated animals than controls. The exception was the 10%W diet (which contained plant oils). Except for the 6%T diet (because of less test-values), treated vs. nontreated values were statistically different Nonautolyzed expired B10 animals (n = 36) were submitted for gross pathological necropsy. Approximately 60% died of neoplasia, with lymphoma/leukemia being the most prevalent; a finding in agreement with that reported by Jackson Laboratory. Of the animals with cancer, equivalent numbers were in the not treated and treated groups. Because of the small number of animals necropsied, association of cancer with different diets could not be assessed.

**Different murine strains.** A/WySn (related to A/J) was the donor of the H-2b mutant H-2a allele of B10.A(4R) mice (to be referred to as 4R). In addition, 4R is H-2 congenic with B10 (H-2a). A/J mice were started on treatment at 180 days of age (n = 9) and 4R mice at 92 days of age (n = 22); both strains were fed the 6%T diet. Untreated A/J (n = 7) and 4R (n = 22) mice were age matched. As shown in Figure 5, mean survival ± SEM of treated (583 ± 28 days) vs. not treated (414 ± 60 days) A/J mice was significantly (p = 0.013) increased 40.8%; median survival of nontreated animals was also extended from 434 to 576 days. In contrast, mean survival of 2-Me treated, 4R mice was not significantly increased (p = 0.17) from that of controls (means of 899 ± 16 for treated vs. 868 ± 27 days for nontreated with medians of 933 vs. 895 days). Survival of 4R mice was slightly less, but not significantly, than that of B10 mice on 6%T diet.

**Discussion**

Since the original reports demonstrating the beneficial effects of 2-Me and other sulfhydryl compounds on cellular functions in tissue culture, reports that weak redox-SH molecules, such as cysteine, D-penicillamine, glutathione, N-acetyl-L-cysteine and alpha-lipoic acid effectively; (a) altered rheumatoid arthritis,56,57 (b) prevented doxorubicin-induced myocardial toxicity,58 (c) restored the thiol deficiency (glutathione) in patients with (i) end-stage diabetic nephropathy,59 (ii) mitochondrial disorders60 and (iii) idiopathic pulmonary fibrosis,61 (d) improved survival of patients with HIV62 presumably via (i) reversing impaired proliferative activity of CD4+ cells,57 (ii) inhibiting HIV entry into CD4+ cells,54 and/or (iii) preventing replication of HIV-1,55,56 (e) reduced the incidence and multiplicity of lymphoma in ataxia telangiectasia deficient mice,63 and (f) attenuated exercise-induced oxidative stress in horses.58 These findings, plus the beneficial in situ aspects of 2-Me found for aging mice and rats,23,38-40 raise the question as to why stronger redox-SH molecules have not been more extensively evaluated for therapeutic value. Is the major obstacle curtailing evaluation of the two strongest, 2-Me and dithiothreitol, their potentially lethal manifestations?37 As results of others and that herein show, all mice and rats treated with 2-Me did not experience toxicity that shortened their lifespan, most likely because the maximum average daily intake of 2-Me was considerably less than the LD50 bolus of 345 ug/gm body weight.37 Instead, there was a significant 12.7% increase in longevity of long-lived, (C57BL x C3H)F1-hybrid mice fed a high fat diet,23 a modest, not significant increase for B10 (on 3 different fat diets) and its H-2 congenic, mutant strain, B10.A(4R), and a significant 40.8% increase for moderately-lived A/J mice; the
And yet over this time-period, there was an average 4.6 gm increase in weight in the absence of 2-Me and only an average 0.5 gm increase in the presence of 2-Me. These differences appear to contrast to that reported for (B57BL x C3H)F1 mice fed the same diet, where those on 2-Me gained more weight than controls at late stages of age. The difference from results herein may be due to different genetics, but most likely was because the F1-hybrids consumed more food on 2-Me than the controls. The difference in food consumption in the two studies appears to be due to the way 2-Me was delivered (water vs. mixed with feed). If the weights at the late ages of 70 and 81 weeks are excluded because of differences in food consumed, a best-fit analysis of the Heidrick, et al. data results in weight changes similar to that found for B10 on this diet.

Obesity is generally manifested because the number of calories consumed is in excess of those expended. The findings herein indicate that prevention of high-fat-diet obesity by 2-Me, at least for B10 mice, was not because they ate less. That leaves two alternatives, (a) physical exercise or energy expenditure was higher by 2-Me-treated mice and/or (b) 2-Me in

| Table 1. Average daily food, water and 2-Me consumed/mouse |
|---|---|---|---|---|---|---|---|
| | -2 Me | +2 Me | -2 Me | +2 Me | -2 Me | +2 Me | -2 Me | +2 Me |
| Water (ml) @ 205 days | 5.2 | 5.6 | 3.7 | 6.2 | 3.8 | 4.2 | 3.0 | 7.0 |
| Food (gms) | | | | | | | | |
| @ 205 days | 3.6 | 3.9 | 4.1 | 3.8 | 3.7 | 3.8 | 3.0 | 3.0 |
| @ 370 days | 3.8 | 3.5 | 3.0 | 3.2 | 3.0 | 3.2 | 3.0 | 2.8 |
| 2-Me (ugm) | 0 | 437 | 0 | 484 | 0 | 328 | 0 | 547 |
| (ugm/gm bw) | 0 | 13.1 | 0 | 15.0 | 0 | 8.5 | 0 | 15.7 |

1Average ugm 2-Me consumed/day/gm body weight at 205 days of age.
some manner altered absorption and/or metabolic utilization of fat. Visual observations are in agreement with Heidrick et al. in that 2-Me-treated mice were more active, but not sufficiently to curtail development of obesity. Thus, even though the mechanism by which obesity was prevented by 2-Me is likely not going to be easily established, alternatives to consider are changes in: (a) intestinal microbiota, (b) factors, such as protamine, (c) duodenal hormones, (d) Carboxypeptidase E expression, (e) feed efficiency similar to that observed for lipoic acid supplementation (a sulfur-containing molecule!) and (f) modulation of food (especially fat) absorption or utilization.

One other important observation made during the present investigation, is that in the last weeks of life on any of the diets, only nontreated mice became recumbent, emaciated, and cachectic. In fact, it was possible to predict via visualization, which nontreated mice would succumb within 48 hours, whereas similar predictions could not accurately be made for treated animals. Such end-stage differences appear relevant for one anti-aging goal, namely, 2-Me treated mice retained a “high quality of life” right up until their death.

Levels of cholesterol associated with obesity were lower in B10 mice treated with 2-Me compared to controls for three of the four diets (the exception was the Wayne breeder diet containing plant oils), suggesting that other diseases associated with obesity may very well be amendable to dietary 2-Me therapy. Necropsy of C57BL/10 mice indicated that the most common causes of death were lymphoma/leukemia.

The mechanism by which 2-Me altered physiological changes associated with aging (see introduction), a process that can be considered a consequence of evolution of organisms from a low oxygen primordial soup, to a land-based, high oxygen environment, is postulated to be via a free radical antioxidant/scavenger, plus “some other means.” Conclusions regarding these postulates need to consider whether the consequence of dietary 2-Me: (a) optimized the participants (cells/factors) of a process prior to their change with age, (b) reversed a process already altered with age, and/or (c) prevented a biochemical or physiological process from ever beginning. There appears to be in situ evidence to support all three of these alternatives. First, optimization is supported by the findings that (a) a single injection of 2-Me given to young mice around the time of antigen sensitization enhanced the in vivo antibody response, and (b) development of obesity in relatively young animals was curtailed. Reverting an age-associated change back to normal is supported by the conversion of the in vivo immune response of old (24 months) C3H mice to that of young (four months) C3H mice by only three or four weekly injections of 2-Me prior to antigen sensitization.

Evidence supporting the prevention of a process from proceeding are: (a) maintenance of dopamine release, (b) slowing tumor formation, (c) curtailing malondialdehyde formation, (d) slowing lipofuscin pigment accumulation as a consequence of autooxidation of PUFA and (e) establishing a mechanism within 28 days of age that resulted in a significant extension of survival of SLE-prone strains (accompanying report). The postulate that 2-Me is acting as an antioxidant/scavenger is based on the quantification of fluorescent lipofuscin pigments defining the amount of free radical damage during aging; i.e., this assay is based on the undegradable polymeric structure of lipofuscin pigments that are not removed by exocytosis. Thus, does the reported delay in lipofuscin accumulation associated with daily 2-Me treatment influence subsequent age related decline in immune activity and/or increased cancer? This is, in part, answered by (a) the reversal of old-age immune function by 2-Me administered days or 3–4 weeks prior to assay without presumably altering lipofuscin levels and (b) the age (81 weeks) at which dramatic tumor differences were noted versus the time at which 2-Me impacted lipofuscin accumulation (95–108 weeks). Better assessment could be made regarding increases in lipofuscin levels relative to decreases in immune function if the actual immune response data were reported, since (a) lipofuscin levels of untreated mice at 110 and 127 weeks were equivalent to those of 2-Me-treated mice at 121 and 134 weeks respectively and (b) yet immune responses of treated and nontreated mice at these “lipofuscin-equivalent” times appear to not be equivalent. Thus, until changes related to continuous 2-Me exposure (extended longevity, slower tumor appearance) are demonstrated to not occur under conditions of early, limited, exposure, they should not be ascribed to 2-Me’s alteration of free radical formation during aging. Thus, the mechanism of action for 2-Me in situ remains unresolved and almost certainly will depend upon which disease process is altered.

Summation. The results presented herein indicate that overt toxicity was not found at an average daily consumption of <16 ug 2-Me per gm body weight. Instead, 2-Me increased longevity of moderately lived mice, prevented high-fat-diet obesity (a major public health concern), and altered one present-day objective of anti-aging research, namely retention of an active quality of life right up until death. Oh, to be a mouse! The findings presented herein raise a number of important questions: (a) By what mechanism(s) does 2-Me impart its benefits and are they different for different diseases? (b) What effect would 2-Me have on animals already obese? (c) Is the prevention of obesity unique to C57BL genetics? And (d) What other processes (diseases) might be impacted by dietary 2-Me? As an interesting aside, to date, the few in situ benefits of 2-Me mimic those achieved with the “anti-aging elixir” resveratrol, raising the possibility that given together they could be a super elixir.

Materials and Methods

Mice and their husbandry. Inbred strains of male and female mice were derived from excesses available from our breeding colony; some male mice were purchased from Jackson Laboratory, Bar Harbor, Maine. Mice were housed in Plexiglas ventilated shoe-box cages (3–4 animals/cage) within a facility that maintained a 12 hour light/12 hour-dark cycle. All animals in the study succumbed from natural causes and had free access to food and to autoclaved water with or without added 2-Me. C57BL/10 mice (n = 36) were submitted to the University of Minnesota Animal Care Laboratory for gross pathological necropsy. All experiments

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were performed in accordance with institutional animal research guidelines and approved by the VA IACUC.

**Water.** Autoclaved distilled/deionized water for drinking was supplied in glass bottles, which were changed and cleaned twice a week. On the day the bottles were changed, 2-Me was added supplied in glass bottles, which were changed and cleaned twice a week. Serum was obtained from a nick of the tail vein and then allowed to clot for six hours in a capillary tube. Serum was then removed. The assay was done at the VA Clinical Diagnostic Laboratory.

**Statistics.** The Mann-Whitney U test was used to assess differences in various parameters of treated vs. nontreated mice. For all comparisons, p values less than 0.05 were considered to be statistically significant.

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**Ethics Statement**

There was no part of the research that inflicted pain or suffering, except that which occurred naturally because of aging. Euthanasia was used to ameliorate suffering of old, incapacitated mice.

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