Prolongation of Life Span and Improved Learning in the Senescence Accelerated Mouse Produced by Aged Garlic Extract

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The effects of aged garlic extract (AGE) on longevity and learning and memory performances were studied in the senescence accelerated mouse (SAM). A solid diet containing 2% (w/w) AGE was given to SAM from 2 months of age. The survival ratio of SAM P8, senescence accelerated animals, treated with AGE was significantly higher than that of untreated controls. AGE, however, did not affect the life span of SAM R1, a senescence-resistant strain. AGE had no effect on body weight and motor activity. In the passive and conditioned avoidance tests, AGE markably improved a memory acquisition process in the step-down and shuttle-box tests, and also a retention process in the step-through and step-down tests in SAM P8. The beneficial effects of AGE were observed in a memory retention process in the step-down test and in an acquisition stage in lever-press test in SAM R1. These results suggest the possibility that AGE might be useful for treating physiological aging and age-related memory deficits in humans.

Keywords aged garlic extract (AGE); life span; learning; memory; senescence accelerated mouse (SAM)

Garlic (*Allium sativum*) has long been used widely not only as a food but also as a nutrient and tonic in folk medicine. In recent years, the effects of garlic have attracted a great deal of attention in pharmacy and medicine. However, chronic feeding of raw garlic causes anemia, weight loss, and failure to grow.[1] Shashikanth *et al.*[2] also reported that long-term feeding of raw garlic extract resulted in decreased bacterial flora in the intestines as well as a reduction in serum globulins. Aged garlic extract (AGE), extracted for more than 10 months, was less irritating and did not produce the above mentioned changes.[1,3] Pharmacological studies of AGE and its components have demonstrated anti-stress effects,[4] a protective action on carbon tetrachloride-induced liver damage,[5] anti-tumor promoting activity against phorbol 12-myristate 13-acetate-induced skin tumor[6] and chemoprevention to 1,2-dimethylhydrazine-induced colon cancer.[7] Considering the low toxicity of AGE after chronic administration and its multiple biological effects, the possibility of the prophylactic use of this drug in aging-related disorders seems an exciting prospect.

The senescence accelerated mouse (SAM)[8] was recently developed in Japan as a genetic animal model for studying aging and spontaneous senescence. Strains were consisted of SAM P (senile-prone strain) and SAM R (resistant strain). SAM P is known to have a very short-life span[8,9] and exhibits various signs[9,10] of senescence in early age, while SAM R is regarded as a reference strain for SAM P. SAM P8,[10] separated from the SAM P strain, has been characterized as exhibiting age-related impairment of learning and memory performances.

In the present study we investigated the effects of AGE, chronically administered in the diet, on longevity and age-related impairment of learning and memory performances in SAM P8 and SAM R1, a substrain of SAM R, using passive and conditioned avoidance tests.

MATERIALS AND METHODS

Animals The substrains of SAM, SAM P8//HS and SAM R1/HS were originally obtained from Professor Toshio Takeda (Kyoto University, Kyoto, Japan) and bred in our laboratory. They were housed individually under conditions of controlled temperature and humidity (22 ± 1 °C, 55 ± 2%). Food (CE-2, Clea Co. Ltd., Tokyo, Japan) and water were provided *ad libitum*.

Extraction of Garlic AGE was generously supplied by the Wakunaga Pharmaceutical Co. Ltd. (Hiroshima, Japan). Sliced raw garlic was extracted with aqueous ethanol for more than 10 months at room temperature. The extract was filtered and concentrated under reduced pressure at a low temperature.

Administration of AGE Male SAM P8//HS and SAM R1/HS were given normal diet (CE-2) until two months of age. Thereafter, SAM P8 and SAM R1 were given continuously the normal diet (P8-Cont; $n=17$ and R1-Cont; $n=12$) or the diet containing 2% AGE (P8-AGE; $n=17$ and R1-AGE; $n=11$) for 9 months (until the final day of the behavioral experiments). Behavioral experiments were conducted when the animals were 11 months old (R1-Cont; $n=11$, R1-AGE; $n=11$, P8-Cont; $n=9$ and P8-AGE; $n=10$). There were no significant differences in food intake between the control and AGE-treated animals in both the SAM P8 and SAM R1 groups.

Behavioral Experiments **1. Passive Avoidance Tests** A) Step-Through Test: The apparatus (Model PA-M1, O'hara Co. Ltd., Tokyo, Japan) consists of a bright and dark compartment which are partitioned by a wall with a round opening in the lower part. The dark compartment was illuminated with a fluorescent lamp and had a grid floor through which an electric foot shock of 36 V AC was applied.[11,12] The step-through test was performed prior to the step-down test every day using the same animals. For the learning trial, a mouse was placed in the bright compartment with its posterior towards the opening for a maximum of 5 min. When the mouse stepped through into the dark compartment, it was given an electric shock and turned back to the bright compartment. The mouse was then immediately returned to its home cage. Starting

on the next day, the mouse was placed in the bright compartment again for a maximum of 5 min every day at the same time of day for 10 d as testing trials. An electric foot shock was always given to a mouse on entering the dark compartment during the period of the testing trials. The latency, which indicates the time elapsed before the mouse entered the dark compartment after it was placed in the bright compartment was recorded, in the learning and first testing trial. The number of mice that did not step through into the dark compartment in the testing trials were recorded every day. At the end of the experiment, the number of error days on which the mice stepped through into the dark compartment, and the cumulative number of mice that succeeded in the task (acquisition rate) and the percentage of mice that kept succeeding in the test, once it was acquired (retention rate) during the repeated testing trials, were also calculated.

B) Step-Down Test: The apparatus consisted of a box with a grid floor (10 × 15 cm, height of walls 40 cm) designed to give a 60 V AC electric shock on touching. A rubber platform (35 mm in diameter at the top, 40 mm in height) was placed in one corner.[11,12] For the learning trial, a mouse was placed gently onto the platform. When the mouse stepped down and touched the grid floor, it was given an electric shock, and jumped back onto the rubber platform. During the latter half of the 10 min period for learning, the number of step-down events and the number of mice that did not step down onto the grid floor were recorded. Starting the next day, the mouse was put on the rubber platform for 3 min every day at the same time of day for 10 d as testing trials. A severe foot shock was always given to a stepped-down mouse over the period of the testing trials. The numbers of step-down events in the learning and first testing trial were recorded. The number of mice that did not step down onto the floor in the testing trials was recorded every day. At the end of the experiment, the number of error days, acquisition rate and retention rate were calculated as in the step-through test.

2. Motor Activity The experimental apparatus is a round tilting-type activity cage, 18 cm in diameter and 18 cm in height (Model SMA-10, O'hara Co. Ltd.). Measurement of motor activity was conducted for 30 min before the learning and testing trials in the passive avoidance tests.[12]

3. Conditioned Avoidance Tests Starting day following the last passive avoidance testing, a shuttle-box test was conducted for 7 d and subsequently a lever-press test for 10 d.

A) Shuttle-Box Test: The shuttle-box type avoidance response was assessed with an apparatus (Model GT-8450, O'hara Co. Ltd.) described previously.[12,13] The temporal parameters were as follows: intertrial interval of 40 s; warning sound and light of duration of 20 s (warning only for the first 10 s and warning with shock of 36 V AC for the next 10 s). Each session consisted of 60 trials/1 h a day. The indices of the avoidance behaviors were the conditioned avoidance response (CAR; the number of avoidance responses during warning period/the number of trials), unconditioned avoidance response (UAR; the number of escape responses during shocking period/the number of trials), error (the number of non-avoidance and non-escape responses/the number of trials) and spontaneous response (SR; spontaneous response observed as irrespective responses to conditioned or unconditioned stimuli/session).

B) Lever-Press Test: The lever-press type avoidance response was assessed with an apparatus (Model GT-8331, O'hara Co. Ltd.) described previously.[12,13] The temporal parameters, intensity of electric shock and indices of avoidance responses were similar to those in the shuttle-box task. In the case of the lever-press test, the number of lever-pressings, irrespective of the conditioned or unconditioned stimuli, was recorded as SR.

Statistics The number of surviving mice (survival ratio) and the number of mice that did not fail in the passive avoidance tests (acquisition and retention rate) were analyzed using the Fisher exact probability test. The step-through latencies, the number of errors in the step-down test were analyzed using the Mann-Whitney *U*-test. For the changes in body weight, motor activity, number of error days in the passive avoidance tests and CAR, UAR, error, and SR data in the conditioned avoidance tests, ANOVA followed by Duncan's multiple range test was employed.

RESULTS

1. Survival Ratio and Body Weight Effects of AGE on the Survival Ratio and Body Weight: The effects of AGE on the survival ratio and body weight of SAM at 11 months of age are shown in Table I. Nearly half (9/17) the P8-Cont group survived to 11 months. The survival ratio of P8-Cont was significantly lower to that of R1-Cont ($p<0.05$). AGE significantly increased the survival ratio of SAM P8 ($p<0.01$), but did not affect that of SAM R1. The body weight of P8-Cont was clearly lower than that of R1-Cont ($p<0.01$). There were no significant differences in body weight between control and AGE-treated animals in both SAM P8 and SAM R1 groups (Table I).

2. Passive Avoidance Tests Effects of AGE on the Step-Through Test: The latencies to enter the dark compartment in the learning trial were not significantly different between the control and AGE-treated animals in both SAM P8 and SAM R1 groups. P8-Cont tended to have a shorter latency than R1-Cont in the first testing trial. A short latency suggests impairment of learning and memory. AGE tended to prolong the latency in both SAM

Table I. Effects of AGE on the Survival Ratio and Body Weight of SAM at 11 Months of Age

Group	Survived/used (*n*/*n*)	Survival ratio (%)	Body weight (g)
R1-Cont	11/12	91.7	34.9±1.2
R1-AGE	11/11	100.0	35.4±1.1
P8-Cont	9/17	52.9#	29.7±0.7##
P8-AGE	16/17	94.1**	29.1±0.8

Solid diet containing 2% AGE was given to P8-AGE and R1-AGE groups from 2 months of age. # $p<0.05$, ## $p<0.01$, between R1-Cont and P8-Cont, ** $p<0.01$, between P8-Cont and P8-AGE. The Fisher exact probability test was used for the survival ratio and Duncan's multiple range test for the body weight (mean±S.E.M.).

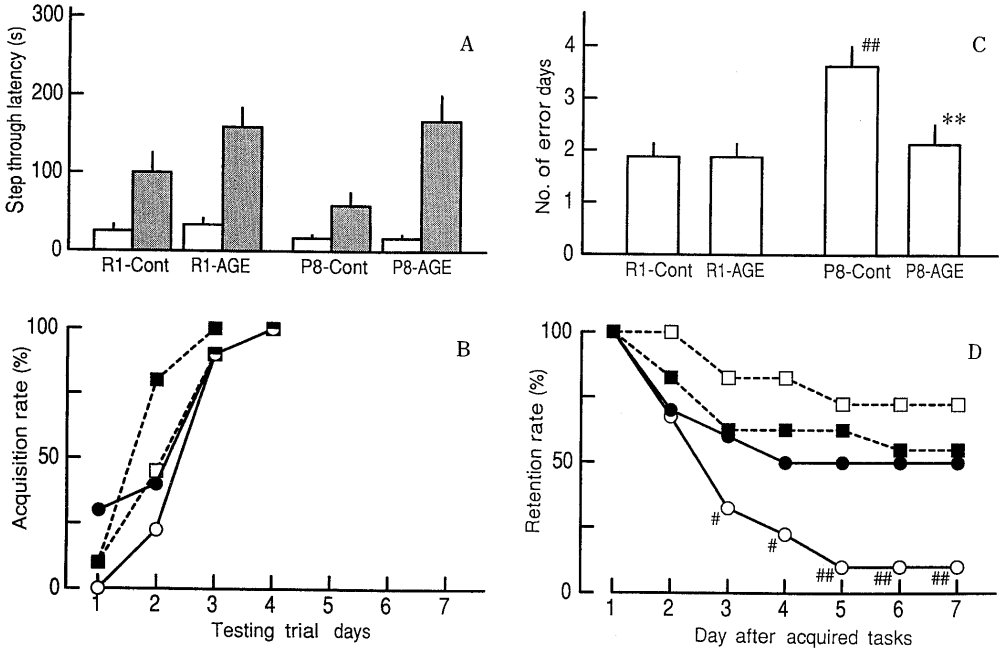

Fig. 1. Effects of AGE on the Step-Through Test in SAM

A, the latency in learning and first testing trials: □, learning trial; ▨, first testing trial. B, the percentage of mice that did not step through into the dark compartment within 5 min (acquisition rate). C, the number of days on which mice stepped through into the dark compartment in the testing trials. D, the percentage of mice that did not make an error after acquisition (retention rate). ○—○, P8-Cont; ●—●, P8-AGE; □---□, R1-Cont; ■---■, R1-AGE. Each value represents the mean ± S.E.M. of the latency and the number of error days, and the computed percentage data of acquisition and retention rates. $^{**}p<0.01$, between the control and AGE groups in the two substrains, $^{\#}p<0.05$, $^{\#\#}p<0.01$, between P8-Cont and R1-Cont, Mann-Whitney *U*-test was used for the latency and the number of error days, while the Fisher exact probability test was used for the acquisition and retention rates.

P8 and R1 animals, but this effect did not reach statistical significance (Fig. 1A). As far as the acquisition rate was concerned, there were no differences between the R1-Cont and P8-Cont group and AGE had no significant effects (Fig. 1B). The total number of error days in the P8-Cont group was higher than that in the R1-Cont group ($p<0.01$). AGE ameliorated the increase in the total number of error days in the SAM P8 group ($p<0.01$), but had no effect in the SAM R1 group (Fig. 1C). The retention rate of the P8-Cont group decreased shortly after the acquisition of task. In contrast, the retention of the P8-AGE, R1-Cont and R1-AGE groups tended to remain higher than that of the P8-Cont group. On the 7th day after the acquisition of task, the retention rates of the P8-Cont, P8-AGE, R1-Cont and R1-AGE groups were 11%, 50%, 73% and 55%, respectively (Fig. 1D).

Effects of AGE on the Step-Down Test: The number of errors were not significantly different between the control and AGE-treated animals in both the SAM P8 and SAM R1 groups in the learning trial. However, the P8-Cont group had a significantly higher number of errors in the first testing trial than the R1-Cont group ($p<0.01$). AGE decreased the number of errors in the SAM P8 group ($p<0.05$), without affecting that in the SAM R1 group (Fig. 2A). The R1-Cont group had acquired the task perfectly by the third testing trial. However, the P8-Cont group showed a significantly slower acquisition rate than the R1-Cont group, and took 7 d to acquire the performance. AGE ameliorated the delay in the acquisition rate in the SAM P8 group, without affecting that in the SAM R1 group (Fig. 2B). The number of error days in the P8-Cont group during testing trials was significantly higher than in the R1-Cont group ($p<0.01$). AGE significantly decreased the number of error days in both substrains (R1; $p<0.05$, P8; $p<0.01$, Fig. 2C). The retention rate of the P8-Cont group rapidly decreased compared with that of the R1-Cont group. AGE did not affect the retention rate in the SAM P8 group, but maintained it at a higher level in the SAM R1 group (Fig. 2D).

3. Motor Activity During the passive avoidance tests, motor activity was measured with a tilting type ambulometer for 30 min every day. The motor activity of the P8-Cont group was higher than that of the R1-Cont group. Chronic administration of AGE as food for 9 months did not affect the motor activities of the two substrains (data not shown).

4. Conditioned Avoidance Tests Effects of AGE on Memory Acquisition in the Shuttle Box Test: CAR of R1-Cont mice gradually increased over ten sessions reaching 95% in the final session (Fig. 3A). However, the percentage of CAR in the P8-Cont group was significantly lower than in the R1-Cont group. AGE continuously and significantly ameliorated the decrease in CAR in the P8-Cont group, without affecting that of the SAM R1 group. The curves of UAR were inversely symmetrical those of CAR. The percentage of UAR in the P8-Cont group was significantly higher than in the R1-Cont group. The UAR of the P8-AGE group was significantly lower than that of the P8-Cont group, while no difference was recorded between the R1-Cont and R1-AGE groups (data not shown). The error was not observed in the shuttle box test. An increase in the SR was observed in the P8-Cont group in the first session, although there were no significant differences in any other sessions between the R1-Cont and P8-Cont groups. AGE did not alter the SR

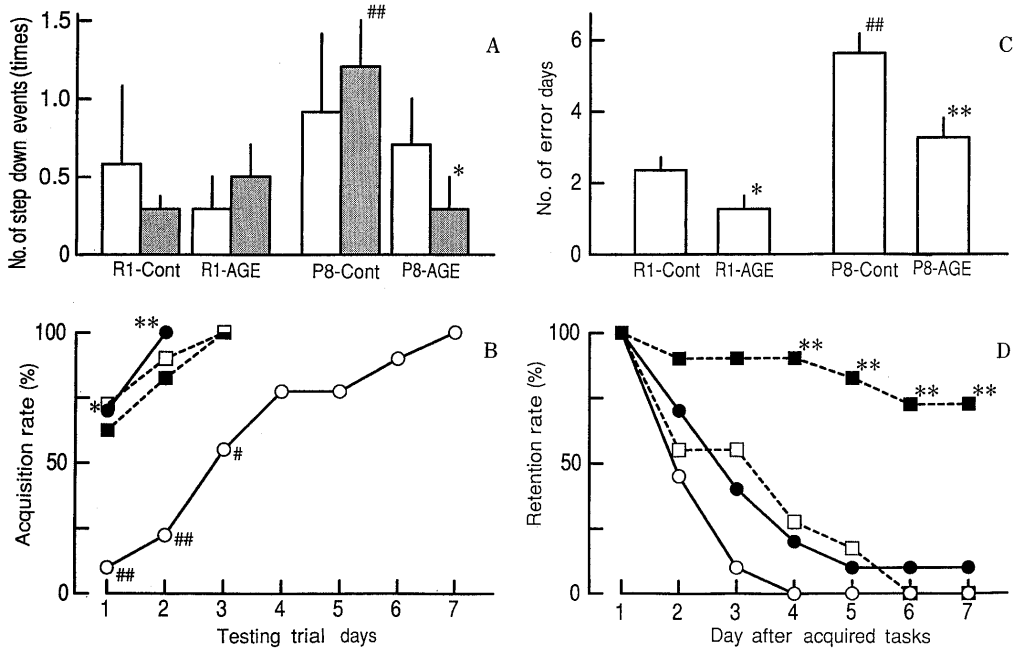

Fig. 2. Effects of AGE on the Step-Down Test in SAM

A, the number of errors in learning and first testing trials: □, learning trial; ▨, first testing trial. B, the percentage of mice that did not step down onto the floor for 3 min (acquisition rate). C, the number of days on which the mice stepped down in the testing trials. D, the percentage of mice that did not make an error after acquisition (retention rate). ○—○, P8-Cont, ●—●, P8-AGE; □---□, R1-Cont; ■---■, R1-AGE. Each value represents the mean ± S.E.M. of the number of errors, the number of error days, and the computed percentage data of acquisition and retention rates. $^{*}p<0.05$, $^{**}p<0.01$, between the control and AGE groups in the two substrains, $^{\#}p<0.05$, $^{\#\#}p<0.01$, between P8-Cont and R1-Cont, Mann-Whitney U-test was used for the number of errors and number of error days, while the Fisher exact probability test was used for the acquisition and retention rates.

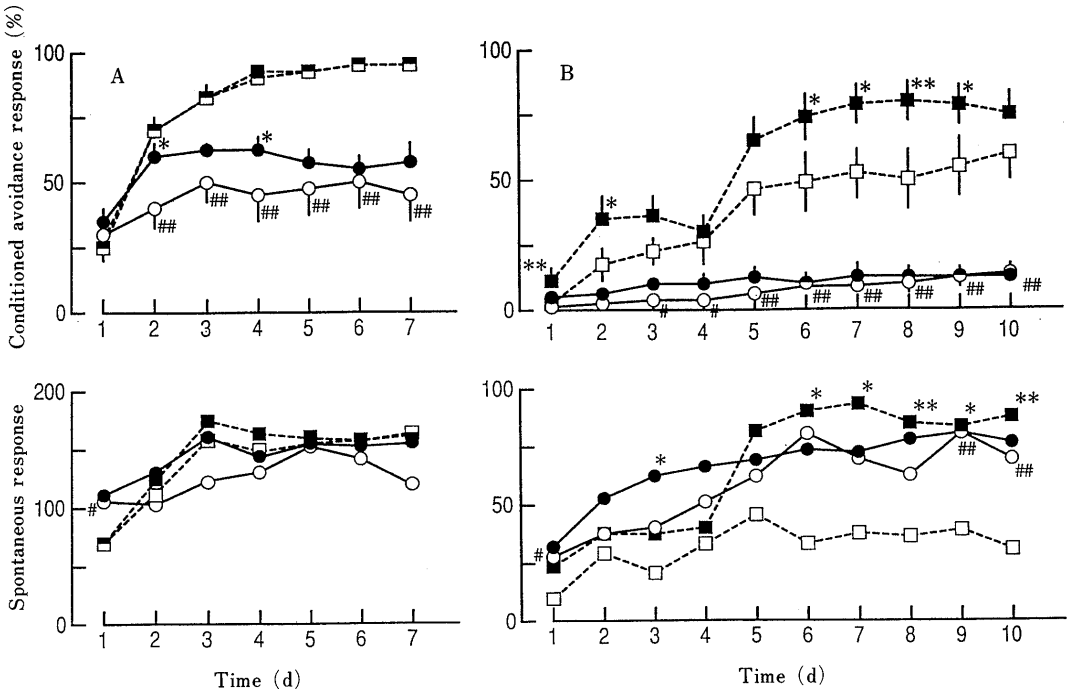

Fig. 3. Effects of AGE on Memory Acquisition and Spontaneous Response in Conditioned Avoidance Tests

Data from the shuttle-box (A) and lever-press (B) tests are shown. Shuttle-box and lever-press tests were performed for 7 and 10 d, respectively. Upper panel, conditioned avoidance response (CAR); lower panel, spontaneous response (SR), see the method. Each value represents the mean ± S.E.M. of CAR and the mean of SR. ○—○, P8-Cont; ●—●, P8-AGE; □---□, R1-Cont; ■---■, R1-AGE. $^{**}p<0.01$, between the control and AGE groups in the two substrains, $^{\#}p<0.05$, $^{\#\#}p<0.01$, between P8-Cont and R1-Cont, Duncan's multiple range test was used.

in the two substrains.

Effects of AGE on Memory Acquisition in the Lever-Press Test The CAR and SR curves in the lever-press test are shown in Fig. 3B. The percentage of CAR was about 60% in the R1-Cont group and only 14% in the P8-Cont group in the final session. AGE significantly increased the CAR

and decreased the UAR in the SAM R1 group, although there was no difference between the P8-Cont and P8-AGE groups (data of UAR not shown). AGE had no effect on the number of errors in the two substrains (data not shown). In the AGE-treated group, a significant increase in SR in the third session in the SAM P8 group was observed and also in the first and after the 5th session in the SAM R1 group.

DISCUSSION

The SAM P8 strain bred under conventional conditions showed an age-associated decrease in the survival ratio and deficits in the ability of learning and memory when compared with the SAM R1 animals at 11 months of age. AGE prevented the decrease in survival ratio in the SAM P8 group, but had no effect on the SAM R1 groups. Food restriction has been known to prolong life span and delay an age-related immune dysfunction in SAM P[14,15] animals and in other rodents.[16-19] In the present investigation, there were no significant differences in food intake and body weight between the control and AGE-treated animals in both the SAM P8 and SAM R1 groups, suggesting that the preventive effects of AGE on aging in the SAM P8 group did not depend on the decreased food consumption but on general metabolic changes.

In the behavioral experiments, AGE decreased the number of error days during testing trials in the step-through test in the SAM P8 group. In the step-down test, AGE also decreased the number of errors and facilitated the acquisition rate in the SAM P8 group. In addition, AGE reduced the number of error days and raised the memory retention rate in the SAM R1 group. In general, one trial is enough for young adult mice to learn the step-down task. The SAM R1 group which we used in this experiment were already nearly one year old and supposed to be considerably aged. An early decline in memory retention in our SAM R1 group might be induced by this physiological aging. The beneficial effect of AGE on memory retention in the SAM R1 group indicates that the preparation improves the memory deficit observed in normally aged subjects. Although AGE facilitated memory acquisition in the SAM P8 group, it did not affect the deteriorated memory retention in the same mice. These results support the idea that memory acquisition and memory retention are based on different mechanisms. Step-through and step-down tests are usually categorized together as passive avoidance tasks. This is also the case for shuttle-box and lever-press tests which are apt to be combined and understood as conditioned avoidance tasks. In our hands, the behavioral profiles of the SAM P8 and SAM R1 groups, as well as the normal diet controls and AGE-treated groups, were not identical when compared in terms of the step-through and step-down tests, and also the shuttle-box and lever-press tests. These differences may be related to the degree of difficulty in performing each behavioral task. Larger numbers of testing trials were required in the step-down and lever-press tests than in the step-through and shuttle-box tests. Therefore we speculate that AGE ameliorated the learning performances in the step-down and lever-press tests to a greater degree than the step-through and shuttle-box tests, according to the relative ease of these tasks.

An increase in lipid peroxides in biological systems has been recognized as one of the most important causes of aging.[20] It has also been well established that lipid peroxides increase[21-24] and the activities of antioxidative enzymes decrease[24,25] in the course of aging. Nomura *et al.*[26] reported that the content of malondialdehyde was significantly higher in the liver and brain of SAM P8 animals compared with SAM R1. The liver of SAM P8 animals has less superoxide dismutase activity than that of SAM R1. AGE and organosulfur compounds present in AGE have an antioxidative effect by protecting the membranes[27,28] and enhancing antioxdative enzyme activity[7,29,30] in peroxidative processes. It has also been reported that dietary antioxidants increase the life-span of mice.[31-33] These results suggest that the antioxidative properties of AGE are involved in its anti-aging effect.

Age-related immune dysfunction, such as a decline in lymphocyte proliferation, is also regarded as a crucial characteristic of the aging process.[34] A significant decrease in splenocyte proliferation induced by phytohemagglutinin[35] and antibody production to challenge by sheep red blood cells *in vivo*[36] were observed in SAM P8 animals. Lau *et al.*[37] reported that AGE and a high molecular protein fraction of AGE increased oxidative burst in murine macrophages, while the protein fraction also enhanced T-lymphocyte proliferation. The protein fraction also enhanced the cytotoxcity and proliferation of human lymphocytes.[38] Furthermore, AGE and its protein fraction potentiate the carbon clearance ability in mice.[39] This evidence suggests that the life-span prolonging effect of AGE in SAM P8 animals may partly come from its immunomodulatory action.

The age-related morphological changes in the SAM P8 brain are summarized as follows: reduction in dendritic spines of the hippocampal pyramidal neurons,[40] amyloid β/A4 protein-like deposition in various regions of the brain,[41] periodic acid Schiff-positive granular structures in the hippocampus[42] and spongiform degeneration of the brain stem.[43] Furthermore, various neurochemical and pharmacological changes have been demonstrated in SAM P8 animals, such as a reduction in *N*-methyl-D-aspartic acid (NMDA) -induced noradrenaline release,[44] a decrease in NMDA receptors in the soma and/or nerve terminals of brain slices,[45] increased glutamic acid and glutamine in the hippocampus and cerebral cortex,[46] a reduction in sensitivity to memory-enhancing doses of cholinomimetics, and enhanced sensitivity to a serotonin antagonist.[47] These findings support the hypothesis that age-related deficits of learning and memory in SAM P8 animals might be related to these morphological, neurochemical and pharmacological changes. We recently found that AGE, as well as a high molecular weight protein fraction of AGE, supported the survival of rat brain neurons in culture (unpublished observation). This suggests the possibility that AGE, and probably its high molecular weight protein fraction, directly prevent age-related changes in the central nervous system, although

further studies are necessary to confirm this.

In conclusion, AGE prolonged the median survival time by slowing down the speed of aging via multiple biological mechanisms, such as antioxidation and immunomodulation, and these two actions may consequently delay the appearance of learning and memory impairment in SAM.

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