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The Amount of Thiolic Antioxidant Ingestion Needed to Improve Several Immune Functions is Higher in Aged than in Adult Mice

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With aging there is an increase of oxidative stress due to an imbalance between the oxidant production and the antioxidant levels in favor of the former. Since immune cell functions are specially linked to reactive oxygen species (ROS) generation, the oxidant/antioxidant balance is essential for these cells. Although low levels of antioxidants cause a decrease in immune function, very high levels of antioxidant compounds could show prooxidant effects. In the present work, we have studied the effect of diet supplementation, for 4 weeks, with two different doses of two thiolic antioxidants, namely thioproline (TP) and *N*-acetylcysteine (NAC), at 0.1% (w/w) and 0.3% (w/w, of each antioxidant) on the main immune system cells, i.e.: macrophages, lymphocytes and natural killer (NK) cells of adult (33 ± 1 week old) and aged (75 ± 1 week old) female Swiss mice. Two groups of animals, adult and aged mice, fed standard diet were used as controls. The results show that the ingestion of 0.1% doses of thiols improves, in the adult mice, several immune functions such as the chemotaxis capacity of both macrophages and lymphocytes, the phagocytosis of macrophages, the lymphoproliferative response to the mitogen Con A and the NK activity. Moreover, no change was observed in adherence capacity of immune cells, and superoxide production was decreased. By contrast, in aged mice the ingestion of these amounts of antioxidants did not change the immune functions studied with the exception of NK activity, which was stimulated. The ingestion of 0.3% of antioxidants by adult mice only increased some immune functions such as adherence and superoxide production, which are markers of oxidative stress. Other functions such as chemotaxis or lymphoproliferative response decreased. However, the ingestion of these very high amounts of thiols by aged animals increased the phagocytosis, the NK activity and specially the lymphoproliferative response to the mitogen, a function that is very depressed with aging.

Keywords: Ageing; ROS; Thiolic antioxidants; Macrophage; Lymphocyte; Diet

INTRODUCTION

Immune cell competence is specially linked to reactive oxygen species (ROS) generation, which is needed to support such important function as the microbicidal and cytotoxic activities.[1] However, excessive amounts of ROS are harmful to immune cells, because their attack to cellular components can lead to cell damage or death. For this reason, the organism, and specially the immunocompetent cells, rely on antioxidant mechanisms in order to maintain the oxidant–antioxidant balance required to preserve the integrity and functionality of membrane lipids, cellular proteins, and nucleic acids, as well as to control signal transduction of gene expression.[2] It seems that immune system cells generally need higher concentrations of antioxidants than other cells.[3] Therefore, the administration of antioxidants may be a useful therapy to improve immune functions. In fact, previous work by us and other authors, has shown an enhancement of leukocyte

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functions after treatment with several antioxidants *in vivo* or *in vitro*.[4–7] This is specially relevant to aging, a process accompanied by changes in functions of immune cells such as phagocytes, NK cells and lymphocytes, specially T cells.[8–11] The deterioration of the immune system with age or "immunosenescence" is believed to contribute to morbidity and mortality, and several studies have suggested a positive association between good immune cell activity, concretely T cell function and longevity.[12] These age-related alterations of the immune functions seem to result from oxidative stress,[2] since aging is linked to an increased rate of free radical generation and decline in antioxidant competence.[8,13,14] For this reason, diet supplementation with antioxidants could prevent or delay the onset of age related immune impairment, and in fact, beneficial effects have been observed in aged subject after that supplementation.[6,15–17] Reduced glutathione (GSH) is one the most important and effective antioxidant defence mechanisms in cells.[18] as well as *N*-acetylcysteine (NAC) and thioproline (TP), which act as glutathione precursors,[19,20] thus exerting a favorable effect on immune functions. GSH has been shown to increase lymphoproliferation in response to mitogens [21] and improve macrophage functions.[4] Further, NAC and TP enhance several functions of leukocytes.[15,22] However, there is some uncertainly on the dose of dietary antioxidants needed to reach an optimal immune function, since high doses of antioxidants, can show prooxidant properties.[5,23,24] Moreover, the amount of antioxidants required to preserve the homeostatic oxidant/prooxidant balance could vary depending on the oxidative state, which changes with the age of the individuals. In the present study in adult and aged Swiss mice we have investigated the effect of two different amounts of NAC and TP, namely 0.1% (w/w) and 0.3% (w/w), ingested for four weeks on the main immune cell functions because in a previous work we observed the improvement of several functions of peritoneal macrophages in adult Swiss mice after the ingestion of 0.1% (w/w) of those antioxidants over the same time period.[22]

MATERIALS AND METHODS

Animals

Female OF1 Swiss mice (*Mus musculus*) (Harlan Interfauna Ibérica, Barcelona, Spain), aged 33 ± 1 and 75 ± 1 weeks old, were maintained at a constant temperature (22 ± 2°C) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France) on a 12 h light/dark cycle and fed Sander Mus pellets (Panlab L.S. Barcelona, Spain) and water *ad libitum.* The animals used did not show any sign of malignancy or other pathological processes.

Reagents

Thioproline and *N*-acetylcysteine were purchased from Sigma (St. Louis, USA), and the following chemicals were also obtained from Sigma: Concanavaline A (Con A) mitogen, formylated peptide (f-Met-Leu-Phe, fMLP), latex beads and nitroblue tetrazolium (NBT). RPMI 1640 medium, fetal calf serum (FCS) and gentamicin were purchased from Gibco (Pasley, Scotland, UK); polycarbonate filters of 3 μm from Millipore (Millipre Iberica, Madrid, Spain) and MIF plates from Kartell (Italy). Trypan blue was from Merck (Darmstadt, Germany).

Experimental Procedure

At 28 and 71 weeks of age, animals were divided into control and treated groups, with 10 animals in each group. One treated group received a diet supplemented with 0.1% (w/w) and another group received 0.3% (w/w) of both *N*-acetylcysteine (NAC) and Thioproline (TP) for 4 weeks. The animals were weighted before and after of treatment. Then, the animals were sacrificed by cervical dislocation according to the guidelines of the European Community Council Directives 86/6091 EEC.

Collection of Leukocytes

Peritoneal suspensions were obtained by a procedure previously described.[25] Briefly, 3 ml of Hank's solution, adjusted to pH 7.4, were injected intraperitoneally, then the abdomen was massaged and the peritoneal exudate cells, consisting of 60% lymphocytes and 40% macrophages, were collected allowing recovery of 90–95% of the injected volume. The cells were counted in Neubauer chambers and then, some samples were adjusted to 5×10^5 macrophages/ml of Hank's medium for the study of phagocytic function, and other samples to 5×10^5 lymphocytes/ml for the assays of adherence and chemotaxis of peritoneal lymphocytes. Then, axillary nodes, spleen, and thymus were removed aseptically and gently pressed through a mesh screen obtaining a cell suspension which was centrifuged to isolate the leukocytes of these organs and then adjusted to 1×10^6 lymphocytes/ml of Hank's medium, in the samples were used for chemotaxis; supplemented medium, RPMI 1640 supplemented with 10% FCS, previously inactivated by heat (30 min at 56°C) and with gentamicin (1 mg/ml), in the samples were used for proliferation assays; and RPMI 1640 without phenol red for cytotoxicity.

Assay of Adherence

The assay of adherence to substrate was carried out by a method previously described.[26] Aliquots of 200 μl of peritoneal, axillary node, spleen or thymus suspensions were placed in eppendorf tubes. At 20 min of incubation, 10 μl were removed from each sample, after gently shaking to resuspend the sedimented cells, and the number of nonadhered lymphocytes was determined by counting in Neubauer chambers (Blau Brand, Germany) in an optical microscope (40× magnification lens). The adherence index, A.I., was calculated as follows:

$$\text{A.I.} = 100 - [(\text{cells/ml supernatant})/(\text{cells/ml original sample})] \times 100.$$

Assay of Chemotaxis

Chemotaxis was evaluated according to a method[26] consisting basically on the use of chambers with two compartments separated by a filter with a pore diameter of 3 μm (Millipore, Madrid, Spain). Aliquots of 300 μl of the different (peritoneal, axillary nodes, spleen or thymus) suspensions were deposited in the upper compartment. Aliquots of 400 μl of the chemoattractant f-Met-Leu-Phe (10^{-8} M), were put into the lower compartment. The chemotaxis index (C.I.) was determined by counting the number of cells in the lower face of the filter.

Assay of Phagocytosis

The phagocytosis assay was carried out by a method described by us.[26] Aliquots of 200 μl or peritoneal suspension in MIF plates, with 20 μl of latex beads (1.09 μm diluted to 1% PBS). The number of particles ingested by 100 macrophages was counted, and expressed as phagocytosis index (P.I.).

Assay of Superoxide Anion Production

The nitroblue tetrazolium (NBT) reduction test, based on an equimolar reaction between NBT and superoxide anion,[27] was carried out for determination of superoxide production according to a method previously described.[26] Briefly, aliquots of 250 μl of peritoneal suspension were mixed with 250 μl of NBT solution (1 mg/ml), and aliquots of 50 μl of latex beads were added to one sample set (stimulated samples) and 50 μl of Hank's medium to the other set (non-stimulated samples). Both, intracellular and extracellular superoxide anion production were evaluated and expressed as nmol/10^6 cells.

Assay of Lymphoproliferation

The proliferation of lymphocytes induced by Con A (1 μg/ml) mitogen, was determined in 72 h cultures. A BrdU labeling and detection commercial kit (Roche Diagnostics, Switzerland) following a method previously described[28] was used. Briefly, it consisted on the addition to the culture medium of BrdU that was incorporated into freshly synthesized DNA. Following fixation of cells, cellular DNA was partially digested by nuclease treatment. Next a peroxidase labeled antibody to BrdU was added and bound to BrdU. At the final step, the peroxidase substrate was added yielding a colored reaction product as the result of peroxidase enzyme activity. The absorbance of the sample (measured at 405 nm) is directly correlated to the level of BrdU incorporated into cellular DNA.

Assay of the Natural Cytotoxicity

The NK activity of the leukocytes from axillary nodes, spleen and thymus was studied, as described by us,[26] using an enzymatic test (Cytotox96 TM, Promega, USA) based on the determination of lactate dehydrogenase (LDH; using a tetrazolium salt). YAC-1 cells from a murine lymphoma were used as target cells and maintained in a complete medium (RPMI 1640 plus 10% FCS). Target cells were seeded in 96 well U-bottom culture plates at 10^4 cells/well in RPMI 1640 without phenol red, and effector cells from axillary nodes, spleen and thymus were added at 10^5 cells/well. The effector/target rate used, 10/1 was found by us to be responsible for similar results to those obtained in previous work with radioactive techniques.[29] Four kinds of control measurements were performed: a target spontaneous release; a target maximum release; an effector spontaneous release; and a volume correction control to adjust the volume change caused by the addition of lysis solution to the maximum release control wells. To determine the percent of target cells killed, the following equation was used:

$$\%\,\text{lysis} = ((E - ES - TS)/(M - ES - TS)) \times 100$$

where E is the mean of absorbances in the presence of effector cells, ES the mean of absorbances of effector cells incubated alone, TS the mean of the absorbances of target cells incubated with medium alone, and M is the mean of maximum absorbances after incubating target cells with lysis solution and subtracting the value from the volume correction control.

Statistical Analysis

The data are expressed as the mean ± SD of the values from the number of experiments shown in the

figures and evaluated statistically by the two-way ANOVA and Scheffe *F*-test for the comparison of parametric samples, $p < 0.05$ being the minimum significant level. The normality of the samples was confirmed by the Kolmogorov–Smirnov test.

RESULTS

No significant difference in the weight data after the antioxidant treatment was observed, neither in adult (40 ± 6 g) nor in old (38 ± 5 g) mice.

The effect of the ingestion of antioxidants (*N*-acetylcysteine plus thioproline) on the main steps of the phagocytic process of peritoneal macrophages are shown in Table I. The supplementation of the diet with 0.1% of each of the two antioxidants increased the chemotaxis and ingestion capacity in the adult mice. No change was observed in adherence capacity, and intracellular superoxide anion production decreased in non-stimulated and increased in stimulated samples. The extracellular measurement of superoxide anion levels showed a decrease in both, non-stimulated and stimulated samples. As regards lymphocyte functions, the statistical analysis rendered no differences in adherence capacity, but chemotaxis improved not only in peritoneum, but also in the three organs studied (Table II). The analysis of the results of lymphoproliferation (Fig. 1) showed that the treatment with antioxidants increased this capacity in cells from axillary nodes, spleen and thymus. The NK activity (Fig. 2) was higher in the treated than in the control group. With respect to the results obtained in the old mice, the thiol ingestion did not influence the macrophage functions, but showed an increase on NK activity in cells from axillary nodes and thymus.

As regards the effect of ingestion of 0.3% of each antioxidant, in the adult animals, adherence underwent a significant increase, whereas chemotaxis and ingestion capacities did not change. On the other hand, the superoxide anion production increased with the treatment, in the intracellular as well as in the extracellular compartment. Lymphocyte functions, such as chemotaxis and lymphoproliferation decreased, but NK of spleen increased significantly after a 0.3% ingestion of thiols. In the old mice, the most evident effect occurred in the ingestion capacity of macrophages, which increased while the other functions of these cells did not change. However, lymphoproliferation and NK activities experimented the main increase as compared to the control group. As regards the other functions, the only change was the significant increase in chemotaxis of cells from axillary nodes.

DISCUSSION

This study shows the beneficial effects of the ingestion of an antioxidant supplemented diet on the immune response, with resulting improvement in several functions of the most relevant immunocompetent cells, namely, macrophages, lymphocytes and cells with natural cytotoxicity. Although it is known that the immune cells need optimal levels of antioxidants for maintenance of their function,[8] and that supplementation with antioxidants may be beneficial,[6,7,22] the present work suggests that the requirements of antioxidants are different depending on the age of the animals. Thus, since a progressive oxidant/antioxidant imbalance is involved in the oxidative stress of aging, even under normal physiological conditions,[8] the amount of antioxidant supplementation required may be higher for the aged than for the adult subjects. In fact, Chandra[30] had already pointed out that the doses of vitamin E needed to improve immune function are greater in aged than in young subjects.

The migration directed to the antigen focus (chemotaxis), which is one of the first steps of the

TABLE I Changes in several functions of peritoneal macrophages caused by *N*-acetylcysteine and thioproline (0.1 or 0.3%, w/w) ingestion in cells from adult and old mice. The data are the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ with respect to the corresponding control. $^{\bullet\bullet}p < 0.01$; $^{\bullet\bullet\bullet}p < 0.001$ with respect to the corresponding value in adults. NS, non-stimulated samples; S, stimulated samples

	Treatments					
	Adult mice			Old mice		
	Control	0.1%	0.3%	Control	0.1%	0.3%
Adherence (A.I.)	42 ± 4	43 ± 6	56 ± 4**	63 ± 8$^{\bullet\bullet\bullet}$	59 ± 10	60 ± 11
Chemotaxis (C.I.)	544 ± 65	907 ± 74***	602 ± 103	131 ± 29$^{\bullet\bullet\bullet}$	132 ± 25	125 ± 23
Phagocytosis (P.I.)	259 ± 45	425 ± 113***	212 ± 49	277 ± 57	289 ± 66	410 ± 81***
Superoxide anion production						
Intracellular NS	14 ± 0.5	7 ± 0.5**	23 ± 3**	30 ± 5$^{\bullet\bullet\bullet}$	33 ± 6	38 ± 7
Intracellular S	30 ± 0.8	46 ± 3***	34 ± 2	46 ± 4$^{\bullet\bullet\bullet}$	48 ± 6	50 ± 7
Extracelular NS	10 ± 0.5	6 ± 0.5*	18 ± 5*	19 ± 2$^{\bullet\bullet}$	17 ± 3	20 ± 2
Extrcellular S	18 ± 2	6 ± 0.5***	27 ± 3*	32 ± 5$^{\bullet\bullet\bullet}$	34 ± 5	26 ± 4

TABLE II Changes in several functions of lymphocytes caused by *N*-acetylcysteine and thioproline (0.1 or 0.3%, w/w) ingestion in cells from adult and old mice. The data are the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ with respect to the corresponding control. $^{\bullet\bullet}p < 0.01$; $^{\bullet\bullet\bullet}p < 0.001$ with respect to the corresponding value in adults

	Treatments					
	Adult mice			Old mice		
	Control	0.1%	0.3%	Control	0.1%	0.3%
Adherence (A.I.)	30 ± 5	36 ± 8	38 ± 4*	60 ± 5$^{\bullet\bullet\bullet}$	61 ± 4	58 ± 7
Chemotaxis (C.I.)						
Peritoneum	789 ± 74	1050 ± 121**	805 ± 112	413 ± 55$^{\bullet\bullet\bullet}$	425 ± 61	450 ± 60
Axillary nodes	1102 ± 92	1574 ± 338*	958 ± 85*	813 ± 81	1018 ± 168*	1281 ± 179***
Spleen	1352 ± 189	1755 ± 373*	655 ± 75***	923 ± 152$^{\bullet\bullet}$	983 ± 174	1033 ± 182
Thymus	2204 ± 280	3003 ± 557**	1436 ± 196**	1099 ± 182$^{\bullet\bullet\bullet}$	1120 ± 223	951 ± 153

immune response,[31] was increased in both, macrophages and lymphocytes from adult mice after ingestion of 0.1% of NAC and TP. These observations are in agreement with the results of previous studies *in vivo*[22,32] showing the immunostimulant action which allows immune cells to reach the infectious focus and to carry out the immune response. However, this dose of 0.1% failed to improve the chemotaxis of our old mice, which was significantly decreased. Previous reports have shown that in oxidative stress conditions such as endotoxic shock[33] or in states with deficient levels of antioxidants,[7] there is a lower chemotaxis. Senescence which shows an oxidative condition,[14] is also accompanied by decreased chemotaxis,[11] and in the present study, the higher dose used, namely 0.3% of both thiolic antioxidants, was needed to improve significantly this function in the aged mice.

Adherence is a key function of immune cells, because in addition to allowing their migration towards inflammatory foci, an adequate adherence is necessary for the cells to reach the immunocompetent organs where the antigens are recognized.[34] Adherence is a function which increases both in oxidative stress situations[33] and in aging.[6,11] This fact is also observed in the present research. Accordingly, recent work shows that free radicals can induce the expression of adhesion molecules.[35] This function was increased by the 0.3% dose of antioxidants in adult mice, which agrees with

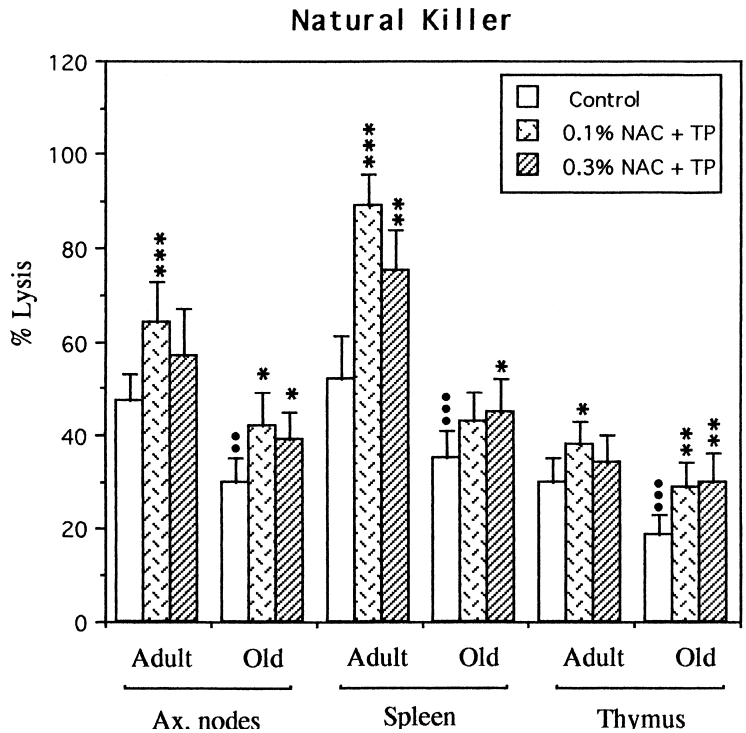

FIGURE 1 Proliferative response of axillary nodes, spleen and thymus lymphocytes to Concanavaline A mitogen of control mice and treated with a diet supplemented with 0.1 or 0.3% of NAC (*N*-acetylcysteine) plus TP (thioproline). The data are the mean ± SD of eight values, being each value the mean of duplicate assays. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ with respect to control. $^{\bullet\bullet}p < 0.01$; $^{\bullet\bullet\bullet}p < 0.001$ with respect to control adult.

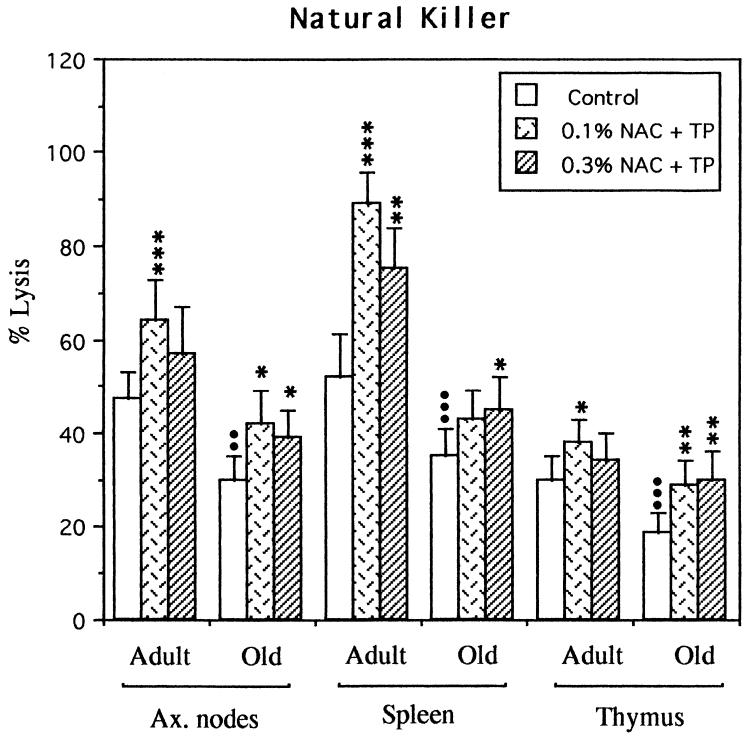

FIGURE 2 Percentage of natural killer (NK) activity of axillary nodes, spleen and thymus lymphocytes from control mice and treated with a diet supplemented with 0.1 or 0.3% of NAC (*N*-acetylcysteine) plus TP (thioproline). The data are the mean ± SD of eight values, being each value the mean of duplicate assays. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ with respect to control. $^{\bullet\bullet}p < 0.01$ $^{\bullet\bullet\bullet}p < 0.001$ with respect to control adult.

previous work showing that antioxidants, such as ascorbic acid[23] and b-carotenes[24] are prooxidants at high concentrations. However, in old animals the high dose of antioxidants did not show any effect.

Particle ingestion (phagocytosis) was significantly stimulated by 0.1% of the thiolic antioxidants in adult mice, as observed in previous studies by using this same supplementation.[22] However, the high dose of 0.3% was not optimal for the adult but it was adequated for restoring the phagocytosis in old mice.

In the present experiments, the thiol compounds at 0.1% caused a decrease in the basal intracellular superoxide anion production in the adult mice. However, in the presence of a phagocytic stimulus there was an increase in that production, which may be considered a favorable response since it raises the microbicidal activity of phagocytes. By contrast, the extracellular production of superoxide, which may be harmful to the organism, was significantly depressed by the thiol compounds in non-stimulated as well as in stimulated samples. Thus, it appears that at the 0.1% concentration these antioxidants improve immune function by raising the cellular microbicidal activity and preventing the production of tissue-damaging oxidants. In these adult mice, at the 0.3% concentration, thiolic compounds increased the basal intracellular as well as extracellular superoxide production, which suggests that at this higher concentration these compounds had a prooxidant effect, thus increasing the formation of free radicals instead of neutralizing them. This observation is in agreement with previous research showing the prooxidant action of high doses of antioxidants.[5,23,24] Interestingly, in old animals, with a higher production of superoxide anion than adult mice, in agreement with previous work,[11] the 0.3% dose of the thiolic compounds did not show any significant effect on the intracellular nor extracellular production of superoxide when administered to the aged mice. This suggests that a marked senescence related disturbance in their oxidant/antioxidant ratio renders the oxygen radical production of immune cells quite unsensitive to the influence of the dietary administration of thiols.

The lymphoproliferative response, one of the most important and representative function of lymphocytes, is stimulated by administration of the thiolic antioxidants at 0.1% to the adult mice, which agrees with previous findings on the positive effects of antioxidants on mitogen induced lymphoproliferation.[2,7,8,15,16] On the other hand, in the present study this function experimented a decrease following the 0.3% thiolic antioxidant administration, probably due to a prooxidant effect of the compound at this high concentration. One of the most important immunological age-related changes is the decrease in mitogen-induced lymphocyte proliferation,[9] which probably results from senescent decrease in the level of reduced glutathione,[36] which is needed for lymphocyte proliferation and activation.[37]

According to our present data, the ingestion of the glutathione precursors, NAC and TP,[19,20] at 0.1% of each could not restore the depressed lymphoproliferation of old mice, but the higher dose (0.3%) reversed the senescent decline in lymphoproliferation. Likewise, our previous work has shown that ingestion of 200 mg/day of vitamin E by aged human subjects improves their depressed lymphoproliferation,[6] and similar results were found in aged women administered supplements of vitamin C and E for 16 weeks[16] and in mice receiving thioproline.[15]

There is senescent decline in the cytotoxic capacity of immune cells, specially of the NK activity,[38] which plays a key role in immune surveillance against tumors, in addition to its important regulatory function.[39] Therefore, it is important that, according to our present results, 0.1% thiol antioxidant supplementation of the diet stimulates the NK activity in both adult and aged mice, but in the aged the most marked improvement is found at the 0.3% dose. In conclusion, the dietary administration of NAC plus TP resulted in an improvement of the immune functions of mice, with the 0.1% dose being the most effective for the adult animals. On the other hand, probably because of their more severely impaired oxidant/antioxidant balance, the aged mice required a 0.3% in order to show optimal immune functions.

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References

[1] Knight, J.A. (2000) "Review: free radicals, antioxidants, and the immune system", *Ann. Clin. Lab. Sci.* **30**, 145–158.
[2] Meydani, S.N., Wu, D., Santos, M.S. and Hayek, M.G. (1995) "Antioxidants and immune response in aged persons: overview of present evidence", *Am. J. Clin. Nutr.* **62**, 1462S–1476S.
[3] Coquette, A., Vray, B. and Vanderpas, J. (1986) "Role of vitamin E in the protection of the resident macrophage against oxidative damage", *Arch. Int. Physiol. Biochem.* **94**, 529–534.
[4] Del Río, M., Ruedas, G., Medina, S., Víctor, V.M. and De la Fuente, M. (1998) "Improvement by several antioxidants of macrophage function *in vitro*", *Life Sci.* **63**, 871–881.
[5] Correa, R., Del Río, M. and De la Fuente, M. (1999) "Improvement of murine immune functions *in vitro* by thioproline", *Immunopharmacology* **44**, 281–291.
[6] De la Fuente, M. and Víctor, V.M. (2000) "Anti-oxidants as modulators of immune function", *Immunol. Cell Biol.* **78**, 49–54.
[7] De la Fuente, M., Carazo, M., Correa, R. and Del Río, M. (2000) "Changes in macrophage and lymphocyte functions in guinea-pigs after different amounts of vitamin E ingestion", *Br. J. Nutr.* **84**, 25–29.
[8] McArthur, W.P. (1998) "Effect of aging on immunocompetent and immflamatory cells", *Periodontol. 2000* **16**, 53–79.
[9] Ginaldi, L., De Martinis, M., D'Ostilio, A., Marina, L., Loreto, M.F. and Quaglino, D. (1999) "The immune system in the elderly II. Specific cellular immunity", *Immunol. Res.* **20**, 109–115.
[10] Pawelec, G., Effros, C., Caruso, C., Remarque, E., Barnett, Y. and Solana, R. (1999) "T cells and aging", *Frontiers Biosci.* **4**, 216–269.
[11] Ortega, E., García, J.J. and De la Fuente, M. (2000) "Ageing modultates some aspects of the non-specific immune response of murine macrophages and lymphocytes", *Exp. Physiol.* **85.5**, 519–525.
[12] Wayne, S.J., Rhyne, R.L., Garry, P.J. and Goodwin, J.S. (1990) "Cell-medicated immunity as a predictor of morbility and mortality in subjects over 60", *J. Gerontol.* **45**, M45–M48.
[13] Meydani, M. (1999) "Dietary antioxidants modulation of aging and immune-endothelial cell interaction", *Mech. Ageing Dev.* **111**, 123–132.
[14] Sastre, J., Pallardó, F.V., García de la Asunción, J. and Viña, J. (2000) "Mitochondria, oxidative stress and aging", *Free Radic. Res.* **32**, 189–198.
[15] De la Fuente, M., Ferrández, M.D., Del Río, M., Burgos, M.S. and Miquel, J. (1998) "Enhancement of leukocyte functions in aged mice supplemented with the antioxidant thioproline", *Mech. Ageing Dev.* **104**, 213–225.
[16] De la Fuente, M., Ferrández, M.D., Burgos, M.S., Soler, A., Prieto, A. and Miquel, J. (1998) "Immune function in aged women is improved by ingestion of vitamins C and E", *Can. J. Physiol. Pharmacol.* **76**, 373–380.
[17] Meydani, M. (2000) "Effect of functional food ingredients: vitamin E modulation of cardiovascular diseases and immune status in the elderly", *Am. J. Clin. Nutr.* **71**, 1665S–1668S.
[18] Saez, G.T., Bannister, W.H. and Bannister, J.V. (1990) "Free radicals and thiol compounds-the role of glutathione against free radical toxicity", In: Viña, J., ed, *Glutathione: Metabolism and Physiological Function* (CRC press, Boca Raton), pp 237–254.
[19] Weber, H.W., Fleming, J.F. and Miquel, J. (1982) "Thiazolidine-4-carboxylic acid, a physiologic sulfhydryl antioxidant with potential value in geriatric medicine", *Arch. Geronotol. Geriatr.* **1**, 299–310.
[20] Miquel, J. and Weber, H.W. (1990) "Aging and increased oxidation of the sulfur pool", In: Viña, J., ed, *Glutathione. Metabolism and Physiological Functions* (CRC press, Boca Raton), pp 187–192.
[21] Pieri, C., Moroni, F. and Recchioni, R. (1992) "Glutathione influences the proliferation well as the extent of mitochondrial activation in rat splenocytes", *Cell. Immunol.* **145**, 210–217.
[22] Blanco, B., Ferrández, M.D., Correa, R., Del Río, M., Guaza, C. and De la Fuente, M. (1999) "Changes in several function of murine peritoneal macrophages by *N*-acetylcysteine and thioproline ingestion. Comparative effect between two strains of mice", *BioFactors* **10**, 179–185.
[23] Otero, P., Viana, M., Herrera, E. and Bonet, B. (1997) "Antioxidant and prooxidant effects of ascorbic acid, dehydroascorbic acid and flavonoids on LDL submitted to different degrees of oxidation", *Free Radic. Res.* **27**, 619–626.
[24] Bowen, H.T. and Omaye, S.T. (1998) "Oxidative changes associated with beta-carotene and alpha-tocopherol enrichment of human low-density lipoproteins", *J. Am. Coll. Nutr.* **17**, 171–179.
[25] De la Fuente, M. (1985) "Changes in the macrophage function with aging", *Comp. Biochem. Physiol.* **81A**, 935–938.
[26] De la Fuente, M., Miñano, M., Víctor, V.M., Del Río, M., Ferrández, M.D. and Miquel, J. (1998) "Relation between exploratory activity and immune function in Aged mice: a preliminary study", *Mech. Ageing Dev.* **102**, 263–277.
[27] Bagasra, O., Howeedy, A. and Kajdacsy-Balla, A. (1988) "Macrophage function in chronic experimental alcoholism. Modulation of surface receptors and phagocytosis", *Immunology* **65**, 405–409.
[28] Medina, S., Del Río, M., Hernanz, A. and De la Fuente, M. (2000) "Age-related changes in the neuropeptide Y effects on murine lymphoproliferation and interleukine-2 production", *Peptides* **21**, 1403–1409.
[29] De la Fuente, M., Ferrández, D., Muñoz, F., De Juan, E. and Miquel, J. (1993) "Stimulation by the antioxidant thioproline

of the lymphocyte functions of old mice", *Mech. Ageing Dev.* **104**, 213–225.
[30] Chandra, R.K. (1997) "Graying of the immune system. Can nutrient supplements improve immunity in the elderly?", *JAMA* **277**, 1398–1399.
[31] Doherty, D.E., Haslett, C., Tonnesen, M.G. and Henson, P.M. (1987) "Human monocyte adherence: aprimary effect of chemotactic factors on the monocyte to stimulate adherence to human endothelium", *J. Immunol.* **138**, 1762–1771.
[32] Correa, R., Blanco, B., Del Río, M., Víctor, V., Guayerbas, N., Medina, S. and De la Fuente, M. (1999) "Effect of a diet supplemented with thioproline on murine macrophage function in a model of premature aging", *BioFactors* **10**, 195–200.
[33] Víctor, V.M., Miñano, M., Guayerbas, N., Del Río, M., Medina, S. and De la Fuente, M. (1998) "Effects of endotoxic shock in several functions of murine peritoneal macrophage", *Mol. Cell. Biochem.* **189**, 25–31.
[34] Mackay, C.R. and Imhof, B.A. (1993) "Cell adhesion in the immune system", *Immunol. Today* **14**, 99–102.
[35] Cheng, J.J., Wung, B.S., Chao, Y.J. and Wang, D.L. (1998) "Cyclic strain-induced reactive oxygen species involved in ICAM-1 gene induction in endothelial cells", *Hypertension* **31**, 125–130.
[36] Stohs, S.J., El-Rashidy, F.H., Lawson, T., Kobayashi, R.H., Wulf, B.J. and Potter, J.F. (1984) "Changes in glutathione and glutathione metabolizing enzymes in human erythrocytes and lymphocytes as a function of age of donor", *Age* **7**, 3–7.
[37] Franklin, R.A., Li, Y.M., Arkins, S. and Kelley, K.W. (1990) "Glutathione arguments *in vitro* proliferative responses of lymphocytes to concanavalin to a greater degree in old than in young rats", *J. Nutr.* **120**, 1710–1717.
[38] Ferrández, M.D., Correa, R., Del Río, M. and De la Fuente, M. (1999) "Effects *in vitro* of several antioxidants on the natural killer function of aging mice", *Exp. Gerontol.* **34**, 675–685.
[39] O'Shea, S.R. and Ortaldo, F. (1992) The biology of natural killer cells insights into the molecular basis of function *The Natural Killer Cell*, (IRL Press, Oxford).