Proceedings of the Nutrition Society (2010), **69**, 651–659 doi:10.1017/S0029665110003848

The 3rd International Immunonutrition Workshop was held at Platja D'Aro, Girona, Spain on 21–24 October 2009

3rd International Immunonutrition Workshop

Session 9: Food ingredients, immunity and inflammation: animal and *in vitro* models
Murine models of premature ageing for the study of diet-induced immune changes: improvement of leucocyte functions in two strains of old prematurely ageing mice by dietary supplementation with sulphur-containing antioxidants

Mónica De la Fuente*
Department of Physiology, Faculty of Biology, Complutense University of Madrid, 28040 Madrid, Spain

Several immune functions are markers of health, biological age and predictors of longevity. A chronic oxidative and inflammatory state is the main cause of ageing and the immune system is involved in the rate of ageing. Thus, several murine models of premature ageing have been proposed owing to their early immunosenescence and oxidative stress, such as ovariectomised rats and mice, obese rats and anxious mice. In the last model, the most extensively studied by us, mice showing anxiety have an aged immune function and redox status as well as a shorter longevity in comparison with animals without anxiety of the same chronological age, being denominated prematurely ageing mice. A confirmation of the above is that the administration of diets supplemented with antioxidants improves the redox status and immune functions and increases the longevity of prematurely ageing mice. Antioxidant precursors of glutathione such as thioproline or N-acetylcysteine, which have a relevant role in ageing, have been the most widely investigated in adult prematurely ageing mice in our laboratory. In the present work, we have studied the effects of the ingestion for 5 weeks of a diet supplemented with 0·1% (w/w) thioproline + N-acetylcysteine on several functions of leucocytes from chronological old (69–73 weeks of age) prematurely ageing mice of two strains (Swiss and BALB/c). The results show an improvement of the immune functions, with their values becoming closer to those in adult animals (24 ± 2 weeks). Thus, an adequate nutrition with antioxidants, even in aged subjects, could be a good strategy to retard ageing.

Ageing: Immunosenescence: Leucocyte functions: Antioxidants

The ageing process and the concepts of biological age and longevity

The ageing process may be defined as a progressive and general deterioration of the functions of the organism that leads to a lower ability to adaptively react to changes and preserve homeostasis. As Strehler pointed out, four rules can define ageing. It is universal (practically all animals suffer ageing), progressive (the rate of ageing is similar at different ages after reaching the adult state), intrinsic (its cause is endogenous) and deleterious (at least for individuals since it leads to their death)[1]. Although the accumulation of adverse changes with the passing of time should not be considered a disease, it strongly increases the risk of disease, and finally results in death.

The ageing process is highly heterogeneous, and thus, there are different rates of physiological changes in the various systems of the organism and in the diverse members of a population of the same chronological age. This justifies the introduction of the concept of 'biological

Abbreviations: GSH, reduced glutathione; NAC, N-acetylcysteine; NK, natural killer; NPAM, non-prematurely ageing mice; PAM, prematurely ageing mice; TP, thioproline.

***Corresponding author:** Professor Mónica De la Fuente, fax +34 913944935; email mondelaf@bio.ucm.es

ageing', which determines the level of ageing experienced by each individual and therefore his/her life expectancy. The biological age is related to the mean longevity, which can be defined as the mean of the time that the members of a population who have been born on the same date live. Subjects of a population with a higher rate of ageing show an older biological age and have a shorter lifespan. Since chronological age fails to provide an accurate indicator of the ageing process, it is necessary to select parameters useful as biomarkers of ageing to find out the rate of ageing and therefore the probable longevity of each subject[2].

An integrated theory of ageing: how, where and why of ageing

Almost 400 single-cause theories have been proposed to explain the ageing process[3], giving only partial explanation for the causes and effects of ageing. Recently, an integrated theory has been published[2] attempting to answer the three important questions of biogerontology: the 'how', the 'where' and the 'why' of ageing. In answer to the question 'how' ageing happens, it is proposed that the ageing process is a chronic oxidative stress condition (increase of oxidant compounds and decrease of antioxidant defences). Thus, it is linked to many age-related changes that affect a large number of parameters including morphology, physiology and behaviour at all levels of organization: molecular, cellular, tissue, organic and that of the whole individual. In addition, since emerging evidence shows the close link between oxidation and inflammation, and since with ageing the pro-inflammatory compounds increase to levels higher than those of the anti-inflammatory compounds, leading to inflammatory stress, an oxidative and an inflammatory state have been suggested as the cause of the loss of function that appears with senescence[2]. To answer 'where' the ageing process starts, it is proposed that this happens in the mitochondria of post-mitotic and differentiated cells. With respect to 'why' ageing happens, the answer seems to be found in several evolutionary theories and related concepts published a long time ago. Hence, ageing is a consequence of characteristics selected by evolution as an advantage for the young subjects of the species allowing them to reach the reproductive age in the best condition and thus preserve the species. However, these characteristics are a disadvantage for old subjects (not needed for species maintenance). An example is the expression of more pro-oxidant and pro-inflammatory genotypes, which allow the reaching of the reproductive age with more probability since the subjects are more able to survive infections. The consequence after adult age is the establishment of what has been called 'oxi-inflamm-ageing'[2].

Immunosenescence: the immune system as a marker of biological age and predictor of longevity

Ageing is associated with an impairment of physiological systems including the immune system, which has evolved to protect individuals against infections and cancers. In fact, with the passage of time there is an increase of infectious and cancerous processes, which exert a great influence on the age-related morbidity and mortality[4,5]. Indeed, the increased death rate found in aged populations is due in great proportion to infections[5,6]. The profound impact of ageing on immunity is presently accepted. Thus, although there are contradictory results, almost every component of the immune system undergoes striking age-associated re-structuring. This leads to changes that may include not only diminished, but also enhanced functions. Therefore, the changes of the immune system with ageing are termed immunosenescence. Despite the rapidly increasing amount of data on immunosenescence in the last few decades[2,7–11], the puzzle of all the changes in the different aspects of the immune function with age has not yet been solved. Nevertheless, the pronounced age-related decrease in T-cell functions is evident, specially in the T-cell helper, which affects humoral immunity and causes an impaired B-cell function[2,7]. In the cells of innate immunity, the phagocytic cells show functions that decrease with ageing and others that are over activated[2,11–13], whereas the anti-tumoral activity of natural killer (NK) cells, in most of the work, shows an age-related decrease[2,11,14].

In addition, it has been demonstrated that the competence of the immune system is an excellent marker of health[2,4,8,15,16] and several age-related changes in immune functions, such as low T-cell proliferative responses, IL-2 secretion and NK cell cytotoxicity, have been linked to longevity[2,15,16]. In previous studies, the earlier-mentioned functions and other immune functions, in lymphocytes and phagocytes, have been established as markers of biological age and therefore as predictors of longevity[2,13,16,17]. These functions have shown similar age-related changes in human subjects (studied from the adult age of 20 until 80, in leucocytes of peripheral blood), and in mice (throughout the life of these animals, with a mean life span of 2 years, in their peritoneal leucocytes)[2]. In order to identify the above parameters as markers of biological age, it is necessary to confirm that the levels shown in particular subjects reveal their real health and senescent conditions. This has been achieved in the following two ways: (A) Ascertaining that the individuals with those parameters showing levels older than those of most subjects of the same population, sex and chronological age, die before their counterparts. This can be confirmed only in experimental animals. (B) Finding that the subjects reaching a very advanced age, preserve these immune functions at levels similar to those of adults. This can be tested on both humans (centenarians) and experimental animals, such as extremely long-lived mice. While biologically older animals showing the immune competence levels characteristic of chronologically older individuals have been found to die prematurely[2,17], centenarians and long-lived mice exhibit a high degree of preservation of several immune functions, which may be related to their ability to reach a very advanced age in a healthy condition[2,13,18–20]. All the above results confirm that the immune system is a good marker of biological age and a predictor of longevity. Moreover, since the evolution of these immune functions is similar in mice and human subjects, it can be assumed that

NS Proceedings of the Nutrition Society

those human subjects showing immune parameters at the levels of older subjects have a higher biological age and a shorter longevity[2].

Neuro-endocrine-immune communication in ageing. The role of the immune system in oxi-inflamm-ageing and in the age-related loss of homeostasis

The immune system does not work alone, since the three regulatory systems, namely the nervous, the endocrine and the immune systems, are intimately linked and interdependent. Thus, there is a 'neuroendocrine–immune' system that allows the preservation of homeostasis and therefore of health[21,22]. The communication between these systems has allowed the understanding of why situations of depression, emotional stress and anxiety are accompanied by a greater vulnerability to infections, cancers and autoimmune diseases, which agrees with the concept that the immune system is affected[2,23,24].

The impairment of physiological systems with ageing especially affects the three regulatory systems and their communication. This seems to justify the loss of homeostatic capacity and the resulting increase of morbidity and mortality that appears with ageing[2,16]. In addition, the age-related changes in the organism are linked to a chronic oxidative and inflammatory stress affecting all cells and especially those of the regulatory systems, which explains their impaired function[2,16]. Thus, immunosenescence has as its basis an oxidative and inflammatory stress situation, and the theory of oxidation–inflammation in ageing, recently proposed[2,16], integrates the idea of 'inflamm-aging'[25] with the oxidation theory of ageing[2,16]. According to this theory, chronic oxidative and inflammatory stress lead to the damage of cell components, including proteins, lipids and DNA, contributing to the age-related decline of physiological functions, especially in cells of the regulatory systems, including the immune system. Moreover, the immune system, due to its capacity of producing oxidant and inflammatory compounds in order to eliminate foreign agents, could be involved with the rate of ageing, increasing oxidation and inflammation, if the age-related oxidative and inflammatory stress are not well controlled[2,16]. In this context, a relationship has been found between the redox and inflammatory state of the immune cells, their functional capacity and the lifespan of a subject. Thus, when an animal shows a high-oxidative stress in its immune cells, these cells have an impaired function and that animal shows a decreased longevity. This happens in chronologically and biologically older human subjects and mice[2,17]. On the contrary, subjects who achieve greater longevity, such as human subject centenarians and extremely long-lived mice, show a preserved redox state and immune functions[2,13,19,20]. One of the most relevant mechanisms involved in the cellular redox state is the NF-κB. This transcription factor plays a key role in regulating the expression of a wide range of oxidants and inflammatory compounds, especially in the immune cells, and increases in many chronic inflammatory diseases. In fact, it has been observed that the NF-κB activation, in resting conditions, is very high in leucocytes from old mice, but lower in extremely long-lived mice and adult animals[19]. Moreover, only old subjects with controlled basal NF-κB activation in leucocytes achieved longevity. Adults with a high basal expression of this factor, died early[19].

In conclusion, only aged individuals who maintain a good regulation of the leucocyte redox state and consequently a good function of their immune cells, with levels similar to those of healthy adults, reach very high longevity. Thus, the immune system seems to be a good predictor of longevity[2,19,20].

Murine models of premature immunosenescence

Support for the above oxidation–inflammation theory of ageing and especially for the role of the immune system in ageing, may be obtained by the study of animal models in which subjects showing premature immunosenescence and a high oxidative and inflammatory stress in their immune cells (and in other cells), show decreased longevity in relation to other members of the group of the same chronological age. In this context, several murine models, such as the following, have been investigated and developed during the last few years as novel approaches to assess premature ageing and the above-mentioned idea.

Menopausal models

Menopausal women as well as ovariectomised rats and mice (a good model for mimicking human menopause) constitute a model for assessing premature ageing, since they show premature immunosenescence and a high oxidative stress condition[2,26–28]. Thus, ovariectomised female rats and mice show a redox state and function in leucocytes similar to those in males[2,27]. In mammalian species, males have a higher oxidative state and a worse function in their immune cells than those of females, having a lower mean life span than the latter[2,27,29]. This fact is due to oestrogens resulting in a less oxidized condition[30].

Obesity models

Obese subjects show a higher incidence of infections and some types of cancer, suggesting an impaired immune function. In general, the scarce studies on the immunity state in obese compared to non-obese subjects of the same chronological age, show a worse immune function, which have been observed in both genetically and diet-induced obese rats[2,31–33]. Moreover, obesity is associated with an inflammatory state[32], and immune cells from obese rats show premature immunosenescence as well as an oxidative stress situation[2,33].

Models of poor response to stress, anxiety and depression

It is accepted that an inadequate response to stress is one of the conditions leading to an acceleration of ageing, accompanied by an impaired immune system and other physiological systems[2,16,17]. Thus, it has been shown that mice with chronic hyper-reactivity to stress and anxiety show a premature immunosenescence, a higher oxidative

https://doi.org/10.1017/S0029665110003848 Published online by Cambridge University Press

stress and a shorter lifespan. These animals show premature ageing[17], and this model will be explained in more detail later. Recently, it has also been observed that mice exposed to the stressful condition of isolation have behavioural responses that reveal a certain degree of depression and a more evident inmunosenescence than control animals of the same age housed in groups[34]. In addition, human subjects suffering chronic anxiety or depression show a significant premature immunosenescence and oxidative stress[23,24].

Model of prematurely ageing mice

A model of premature ageing in mice based on altered stress-related behavioural response and immunosenescence has been established[17]. The animals are termed prematurely ageing mice (PAM), in contrast to the non-prematurely ageing mice (NPAM) of the same population, sex and chronological age, are identified by their poor response in a simple T-maze exploration test. This provides strong support for the concept that the nervous and the immune systems are closely linked. In mice showing premature ageing, it has been observed that several immune functions were similar to those of chronologically older mice. In addition to a more significant immunosenescence, the PAM exhibited high levels of anxiety and emotionality, showing a brain neurochemistry characteristic of older animals and an oxidative stress situation. Moreover, PAM also had increased baseline corticosterone and a blunted stress response when compared to NPAM. Nevertheless, the most convincing evidence that the immune function parameters studied are useful markers of biological age, is that the PAM showed a shorter lifespan than their NPAM counterparts [2,16,17].

Effects of a diet supplemented with antioxidants in ageing and immunosenescence

Ageing cannot be 'eliminated', it can only be mitigated, i.e. making the process slower. Since the base of the functional longevity of each subject is health maintenance, and this depends on the genes (approximately 25 %) and on the lifestyle and environmental factors (75 %), it is possible to retard the rate of ageing through the modulation of these factors such as nutrition[2]. Among all the aspects that can be considered good nutrition, the antioxidant compounds present in the diet seem to be the most effective. As mentioned above, ageing is the result of a chronic oxidative stress with an oxidant–antioxidant imbalance due to an excess of the oxidants and a decrease or impairment of the antioxidant defences[2]. In fact, a confirmation of this is the age-related decrease of several antioxidants[26,35] as well as the increase of longevity in animals that received these antioxidants in their diet[36,37].

Moreover, nutritional status has a marked effect on immune response in elderly individuals[38]. Since the functional state of the immune system is a marker of health, biological age and a predictor of longevity, it would be convenient to test the effects of a strategy such as diets rich in antioxidant compounds to retard the ageing process, analysing immune cell functions and their redox state as well as the mean longevity of the subjects. The administration of antioxidants could prevent the age-related imbalance of oxidants–antioxidants in the immune cells. Nevertheless, it should be considered that the immune cells need to produce oxidants to carry out their functions and thus, they may exhaust their reserves of antioxidants[2]. This could help to explain why, in both adult experimental animals and human subjects, the functional capacities of the immune cells improve after diet supplementation with the appropriate amount of antioxidants[2]. It is evident that the amount of antioxidants needed by the immune cells in old subjects is higher than that in adults, since these cells show an age-related impairment of redox regulation with a higher production of oxidants and lower levels of antioxidant defences[2,39]. Thus, the administration of compounds such as vitamins C and E, polyphenols and precursors of reduced glutathione (GSH; taurine, thioproline (TP) and N-acetylcysteine (NAC), among others) in isolation, in nutritional formulations or through food rich in these compounds, has been shown to improve the immune functions and decrease the oxidative stress in leucocytes and in other cells of the organism[2]. These effects have been shown in adults, but especially in prematurely ageing subjects and in chronologically elderly men, women and mice[2,11,16,26]. Moreover, the confirmation of the central role of the immune system in oxi-inflamm-ageing is that the administration of adequate amounts of antioxidants in the diet, increases the longevity of the subjects[2]. This has been observed in experimental animals such as mice with a lifespan of 2 years. Since the improvement in the immune function and redox state found with antioxidant supplementation is similar in human subjects and mice, and because these changes in mice are accompanied by an increase in lifespan, it is probable that similar effects could be observed in human subjects. These antioxidants seem to have a direct effect on the immune cells since they improve the same immune cell and redox parameters *in vitro* as well as *ex vivo* after their ingestion in the diet[2].

The effects of the dietary supplementation with antioxidants on immune cell functions and their redox state have been observed in several of the models of premature ageing mentioned earlier. The results found with mice suffering anxiety, the premature ageing model previously mentioned, will be discussed in the next section. In the murine model of ovariectomy, an improvement of several immune functions by a five-week ingestion of a diet enriched (1 mg/mouse/d) in soyabean isoflavones and green tea has been observed in ovariectomised old mice[40]. This agrees with the overall observation that any positive change in the diet is more effective in improving immune response in subjects of a biological older population[2,16,17].

Effects of a diet supplemented with antioxidants on a model of prematurely ageing mice

In the model of PAM the effect of a diet supplemented with appropriate amounts of antioxidants on many

https://doi.org/10.1017/S0029665110003848 Published online by Cambridge University Press

immune functions and oxidative stress parameters, which were previously accredited as markers of biological age, has been extensively studied[17]. A dietary supplementation of biscuits enriched with nutritional doses of vitamin C and E, zinc, selenium and β-carotenes, for 15 weeks, with both 5% and 20% (w/w), improves the function and redox balance of peritoneal immune cells from chronologically adult (27–31 weeks of age) and mature (48–52 weeks of age) PAM and NPAM animals. However, the effects were stronger in PAM, and 20% supplementation was more effective than 5%[17,41]. This supplementation with 20% of biscuits enriched with antioxidants also improved the functions and redox balance of the immune cells from chronologically young (16–20 weeks of age) PAM after only 5 weeks of ingestion[42,43]. Moreover, a supplementation with 20% (w/w) of polyphenol-rich cereals, for 5 weeks, improved the immune cell functions in young (16–20 weeks of age) PAM[44].

The type of antioxidant supplementation most frequently studied in PAM and NPAM has been that using sulphur-containing antioxidants that are precursors of GSH[17]. These antioxidants have been shown to increase longevity[36,37]. GSH is the most abundant non-protein thiol in mammalian cells, being considered essential for their survival, and with an important role in many biological processes[2]. An increase in the levels of GSH reinforces antioxidant protection, preserves the intracellular redox state and the cellular function[2,26,35]. Therefore, optimal immune functions, such as T-cell proliferation among others, will require proper levels of GSH[2,26,35]. In previous studies, it has been observed that GSH stimulates many immune functions *in vitro* and protects cells against apoptosis[2]. The two antioxidant GSH precursors most often studied and used in the present work have been TP and NAC. TP is an antioxidant present in mitochondria, which can increase the levels of GSH[37] and thus increases the longevity[36,37]. Although this is an aspect very little studied, in previous investigations, it has been shown that TP *in vitro* improves several functions of immune cells from mice, as well as the activity of antioxidant enzymes[2]. In aged mice, the supplementation with TP (0·07 (w/w), for 5 weeks) improved several immune functions[45]. NAC is an antioxidant that acts as a GSH precursor[26,35] and also neutralizes free radicals in a direct manner. This antioxidant action has been observed in immune cells from mice with endotoxic shock, a situation of high oxidative stress[46]. NAC *in vitro* increases several functions of peritoneal macrophages from adult mice in a similar way to GSH, as well as the activity of antioxidant enzymes[2]. In elderly women, the ingestion of NAC improves several immune functions and the redox state[26].

In adult PAM, the supplementation of a diet with TP (0·1% (w/w) for 5 weeks) improves the peritoneal macrophage functions[47] and the same occurs with the supplementation with NAC[48]. When the diet contains both TP and NAC (0·1% (w/w)), the supplementation for 4–5 weeks improves the function of immune cells in Swiss and BALB/c mice[49,50].

Effects of a diet supplemented with two sulphur-containing antioxidants (thioproline and N-acetylcysteine), precursors of reduced glutathione, on several leucocyte functions in old prematurely ageing mice of two strains

In previous studies, it has been shown that the ingestion of a diet supplemented with TP+NAC (0·1% (w/w)) by adult female Swiss and BALB/c mice for a short period of time (4–5 weeks) improves several immune functions such as chemotaxis, lymphoproliferative response to the mitogen concanavalin A, IL-2 release and NK activity in leucocytes from peritoneum, axillary nodes, spleen and thymus[39,49]. In the present work, it has been investigated whether this supplementation with 0·1% of TP+NAC for 5 weeks could be enough in chronologically old (69–73 weeks of age) PAM Swiss and BALB/c mice to improve those immune functions to the level of adult (22–26 weeks of age) animals.

Female mice of the Swiss and BALB/c strains (Harlan, Iberica, Spain) 20–24 weeks of age were maintained in sterile conditions at a constant temperature (20–24°C) on a 1212 reversed light–dark cycle and fed water and Standard Sander Mus pellets (A.04 diet; Panlab LS, Barcelona, Spain) *ad libitum* until the moment of starting the experiment. At 64–68 weeks of age, the exploratory behaviour of each mouse was tested in a T-shaped maze. As previously described[17], the mice that completed the exploration of the first arm of the maze four times in more than 20 s (once each week for 4 consecutive weeks) are considered PAM and those that spent less than that time are NPAM. At 69–73 weeks of age, eight groups of 10 animals each were used. In each strain, 10 PAM and 10 NPAM received a diet (A.04 diet; Panlab) supplemented with 0·1% (w/w) of both TP and NAC (Sigma, San Louis, MO, USA) for 5 weeks (PAMA and NPAMA, respectively). Two other groups of 10 PAM and 10 NPAM, of both strains, received a normal diet (PAMC and NPAMC, respectively) during that time. After 5 weeks, the mice were killed by cervical dislocation according to the European Community Council Directives 86/6091 EEC and the axillary nodes, spleen and thymus were removed. In parallel, a group of adult (20–26 weeks of age) Swiss and BALB/c mice were sacrificed.

The leucocyte suspensions from organs were obtained and the functions studied were evaluated following methods previously described[49]. The chemotaxis was evaluated using a chamber with two compartments separated by a filter. The samples of leucocytes were deposited in the upper compartment and the chemoattractant (f-met-leu-phe, 10^{-8} mol/l) in the lower compartment. After 3 h incubation, the filters were fixed and stained and the number of leucocytes found in four scans of 5 mm each on the lower face of the filter was determined and nominated the chemotaxis index. The proliferation of lymphocytes was determined using a commercially available 5-bromo-2′deoxyuridine ELISA (BrdU labelling and detection kit III; Boehringer, Mannheim, Germany). The leucocytes were incubated for 48 h in the absence (basal proliferation) or presence of the mitogen concanavalin A (1 mg/ml; Sigma). The absorbance of the samples (measured at

https://doi.org/10.1017/S0029665110003848 Published online by Cambridge University Press

Table 1. Effect of a diet supplemented with two sulphur-containing antioxidants (thioproline (TP) and N-acetylcysteine (NAC); 0·1% (w/w) for 5 weeks) on several functions of axillary node leucocytes from chronologically old prematurely and non-prematurely aged Swiss and BALB/c mice
(Mean values and standard deviations for 10 subjects)

	NPAMC		NPAMA		PAMC		PAMA		AC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Swiss mice										
Chemotaxis	441	91^{c}	934	167***	260	64^{c}†	808	187***	860	120
Basal proliferation (absorbance)	0·18	0·02	0·17	0·05	0·15	0·05^{a}	0·16	0·02^{b}	0·21	0·03
Proliferation to Con A (absorbance)	0·36	0·06^{c}	0·50	0·09***	0·25	0·06^{c}†	0·62	0·06***	0·61	0·13
NK activity (lysis %)	28	6^{c}	36	4**	22	4^{c}	35	5**	44	5
IL-2 release (pg/ml)	152	39^{c}	210	36**	108	20^{c}†	143	40*	329	57
BALB/c mice										
Chemotaxis	474	106^{c}	898	158***	257	45^{c}††	638	161***	782	111
Basal proliferation (absorbance)	0·08	0·02^{b}‡‡	0·09	0·03	0·07	0·01^{b}‡‡	0·07	0·01	0·17	0·03‡
Proliferation to Con A (absorbance)	0·33	0·04^{c}	0·46	0·09**	0·19	0·05^{c}††	0·26	0·04**	0·58	0·10
NK activity (lysis %)	25	3^{c}	41	6***	20	3^{c}†	34	4***	58	7‡
IL-2 release (pg/ ml)	226	24^{c}‡‡	565	62***	149	23^{c}†‡	727	133***	418	66‡

NPAM, non-prematurely ageing mice; PAM, prematurely ageing mice; NPAMC and PAMC, NPAM and PAM controls; NPAMA and PAMA, NPAM and PAM with antioxidant supplementation; AC, adult controls; Con A, concanavalin A; NK, natural killer.
The data were analysed statistically by the three-way ANOVA for unpaired observations, followed by the Scheffe's F *post-hoc* test. *$P<0·05$; **$P<0·01$; ***$P<0·001$ with respect to the corresponding values in controls (NPAMC and PAMC). $^{a}P<0·05$; $^{b}P<0·01$; $^{c}P<0·001$ with the corresponding values in adults. †$P<0·05$; ††$P<0·001$ with respect to the corresponding values in NPAMC. ‡$P<0·05$; ‡‡$P<0·01$ with respect to the corresponding values in Swiss mice.

Table 2. Effect of a diet supplemented with two sulphur-containing antioxidants (thioproline (TP) and N-acetylcysteine (NAC); 0·1% (w/w) for 5 weeks) on several functions of spleen leucocytes from chronologically old prematurely and non-prematurely aged Swiss and BALB/c mice
(Mean values and standard deviations for 10 subjects)

	NPAMC		NPAMA		PAMC		PAMA		AC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Swiss mice										
Chemotaxis	439	91^{c}	811	102***	343	80^{c}†	670	167***	718	139
Basal proliferation (absorbance)	0·19	0·01^{b}	0·20	0·05	0·16	0·02^{c}††	0·32	0·06***	0·23	0·04
Proliferation to Con A (absorbance)	0·40	0·08^{b}	0·41	0·07	0·22	0·05^{c}†††	0·46	0·09***	0·54	0·07
NK activity (lysis %)	21	3^{c}	28	3***^{b}	18	4^{c}	26	3***^{b}	45	8
BALB/c mice										
Chemotaxis	478	106^{c}	915	160***	282	93^{c}††	802	188***	874	175
Basal proliferation (absorbance)	0·12	0·04^{c}‡‡	0·13	0·03^{b}‡‡	0·12	0·03^{c}‡‡	0·16	0·06‡	0·20	0·03
Proliferation to Con A (absorbance)	0·31	0·07^{b}‡	0·50	0·12**	0·21	0·07^{c}††	0·46	0·10***	0·43	0·07‡‡
NK activity (lysis %)	25	5^{c}	38	5***^{b}‡	16	2^{c}†††	30	3***	60	11‡‡‡

NPAM, non-prematurely ageing mice; PAM, prematurely ageing mice; NPAMC and PAMC, NPAM and PAM controls; NPAMA and PAMA, NPAM and PAM with antioxidant supplementation; AC, adult controls; Con A, concanavalin A; NK, natural killer.
The data were analysed statistically by the three-way ANOVA for unpaired observations, followed by the Scheffe's F *post-hoc* test.*$P<0·05$; **$P<0·01$; ***$P<0·001$ with respect to the corresponding values in controls (NPAMC and PAMC). $^{b}P<0·01$; $^{c}P<0·001$ with the corresponding values in adults. †$P<0·05$; ††$P<0·01$; †††$P<0·001$ with respect to the corresponding values in NPAMC. ‡$P<0·05$; ‡‡$P<0·01$; ‡‡‡$P<0·001$ with respect to the corresponding values in Swiss mice.

405 nm, with a reference wavelength of 490 nm) is directly correlated with the level of 5-bromo-2′deoxyuridine incorporated into cellular DNA. The NK activity of the leucocytes was studied using an enzymatic colorimetric assay (Cytotox 96, Promega, Madison, WI, USA) for cytolysis measurements of target cells (yeast artificial chromosome-1 cells from a murine lymphoma) based on the determination of lactate dehydrogenase enzymatic activity using tetrazolium salt. After 4 h of incubation, the percentage lyses of target cells was calculated. The concentration of IL-2 was determined in culture supernatants of leucocytes from axillary nodes after 48 h of incubation with concanavalin A using an ELISA kit (R&D System, Minneapolis, MN, USA).

The results are shown in Tables 1–3 for leucocytes from axillary nodes, spleen and thymus, respectively. The chemotaxis indexes of leucocytes from axillary nodes, spleen and thymus (with similar values in both strains of mice) were in Swiss and BALB/c PAMC, smaller than in the corresponding NPAMC. In all cases, the values in old PAM and NPAM were lower than those in the adult animals. The ingestion for 5 weeks of a diet supplemented with TP+NAC increased, in general, with statistical significant differences, the chemotaxis in PAMA and NPAMA with respect to the corresponding controls (PAMC and NPAMC). Moreover, the ingestion of the antioxidants brought the levels of chemotaxis in aged animals close to those in adults.

NS Proceedings of the Nutrition Society

https://doi.org/10.1017/S0029665110003848 Published online by Cambridge University Press

Table 3. Effect of a diet supplemented with two sulphur-containing antioxidants (thioproline (TP) and N-acetylcysteine (NAC); 0·1 % (w/w) for 5 weeks) on several functions of thymus leucocytes from chronologically old prematurely and non-prematurely aged Swiss and BALB/c mice (Mean values and standard deviations for 10 subjects)

	NPAMC		NPAMA		PAMC		PAMA		AC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Swiss mice										
Chemotaxis	350	58^{c}	756	118***	248	67^{c}	635	180***	550	140
Basal proliferation (absorbance)	0·13	0·02	0·15	0·03	0·08	0·02^{b}††	0·14	0·02***	0·12	0·03
Proliferation to Con A (absorbance)	0·20	0·05^{b}	0·28	0·08*	0·12	0·04^{c}†††	0·22	0·06**	0·31	0·05
NK activity (lysis %)	14	3^{c}	24	3***^{a}	11	2^{c}†	23	3***^{a}	32	5
BALB/c mice										
Chemotaxis	311	49^{c}	402	85	148	45^{c}	367	99***	620	30
Basal proliferation (absorbance)	0·07	0·01^{c}‡‡‡	0·10	0·03**	0·07	0·01^{c}	0·08	0·02^{b}‡‡	0·16	0·02‡
Proliferation to Con A (absorbance)	0·12	0·04^{c}‡‡	0·31	0·07***	0·08	0·03^{c}†‡	0·15	0·03***^{b}‡	0·35	0·07
NK activity (lysis %)	21	4^{c}‡‡	28	3**^{b}	14	3^{c}†††‡	29	3***^{b}‡	67	8‡‡‡

NPAM, non-prematurely ageing mice; PAM, prematurely ageing mice; NPAMC and PAMC, NPAM and PAM controls; NPAMA and PAMA, NPAM and PAM with antioxidant supplementation; AC, adult controls; Con A, concanavalin A; NK, natural killer.
The data were analysed statistically by the three-way ANOVA for unpaired observations, followed by the Scheffe's F *post-hoc* test. **$P<0·01$; ***$P<0·001$ with respect to the corresponding values in controls (NPAMC and PAMC). $^{a}P<0·05$; $^{b}P<0·01$; $^{c}P<0·001$ with the corresponding values in adults. †$P<0·05$; ††$P<0·01$; †††$P<0·001$ with respect to the corresponding values in NPAMC. ‡$P<0·05$; ‡‡$P<0·01$; ‡‡‡$P<0·001$ with respect to the corresponding values in Swiss mice.

With respect to basal proliferation, there are differences between Swiss and BALB/c mice, the latter showing lower values. In general, the levels of proliferation in PAMC and NPAMC were lower than those in adults. The supplementation only increased the basal proliferation in cells from spleen and thymus of PAMA. Nevertheless, the proliferative response of lymphocytes to concanavalin A, which was lower in all cases in PAMC with respect to the corresponding NPAMC and also in all the old NPAMC and PAMC with respect to the values in the corresponding adults, increased after the supplementation. Moreover, the values in aged PAMA and NPAMA were generally similar to those in adults. The NK activity was, in general, smaller in PAMC with respect to NPAMC and in all cases in PAMC and NPAMC when the values were compared with those corresponding in adults. The ingestion of a supplementation of sulphur-containing antioxidants increased the NK activity in all cases bringing the values closer to those in adults although they did not reach those values. The IL-2 release in lymphocytes from axillary nodes (Table 1) was higher in cells from BALB/c mice than in those from Swiss. In both Swiss and BALB/c PAMC groups, there were significant decreases in the levels of this cytokine with respect to those in NPAMC, and in all groups of old animals (both NPAMC and PAMC) the values were lower than in adults. After the antioxidant supplementation in all the groups, there were significant increases of IL-2 concentrations bringing them close to the levels of adults, a change that was more evident in BALB/c mice.

Since all the functions studied are markers of biological age in mice and predictors of longevity[2], the present data show that the ingestion of a low amount (0·1 % (w/w)) of two antioxidants such as TP+NAC for a short term (5 weeks) decreases the biological age of mice making it more similar to that of chronological adults. Moreover, this effect was found in two different strains of mice, outbred Swiss and inbred BAB/c mice, and in chronologically old PAM and NPAM, but more extensively in PAM. In a previous study, it has been observed that in chronologically adult PAM and NPAM this kind of supplementation was also more effective in PAM than in NPAM, and in Swiss mice than in BALB/c mice[49]. However, in adult mice, the supplementation was, in general, less effective than in old animals, since the present results show that practically all the functions studied in leucocytes have been improved after the ingestion of the diet supplemented with the antioxidants (Tables 1–3). Although in chronologically old Swiss mice an ingestion of a high concentration of these antioxidants (0·3 %) caused a stimulation of several of the functions studied here, this concentration in the adults decreased those functions and increased the oxidative stress of the animals[39]. In the present study, the mice were chronologically old, but they were divided into two groups with different biological age, since NPAM are always biologically younger than PAM of the same age[17]. Based on the present results, 0·1 % (w/w) TP+NAC seems an appropriate amount to improve immune function in aged mice. Nevertheless, further research on the effects of higher amounts of those antioxidants in old PAM and NPAM should be carried out.

Conclusion

A similar diet to that used in the present work, also ingested for 5 weeks, improved several peritoneal macrophage functions in chronologically adult Swiss mice, in NPAM and especially in PAM[50], with these animals showing an increased longevity (N Guayerbas and M De La Fuente unpublished results). Since the improvement of the immune functions studied in peritoneal leucocytes after antioxidant supplementations is very similar to that found in the immune cells from organs such as axillary nodes, spleen and thymus, it is possible that the immune 'rejuvenation' found in the present work could allow an increase of the longevity of animals. Thus, the ingestion of a diet with adequate concentrations of antioxidants, even in chronologically and biologically aged subjects, seems to be

https://doi.org/10.1017/S0029665110003848 Published online by Cambridge University Press

a good strategy to maintain health and retard the inevitable ageing process.

Acknowledgements

This work was supported by the following grants: MEC (BFU 2005-06777), MICINN (BFU2008-04336), UCM Research Group (910379ENEROINN) and RETICEF (RD06/0013/0003) (ISCIII) of Spain. The author declares no conflict of interest.

References

1. Strehler BL (1977) *In Time, Cells and Aging*. New York: Academic Press.
2. De la Fuente M & Miquel J (2009) An update of the oxidation–inflammation theory of aging: the involvement of the immune system in oxy-inflamm-aging. *Curr Pharm Des* **15**, 3003–3026.
3. Medvedev ZA (1990) An attempt at a rational classification of theories of aging. *Biol Rev* **65**, 375–398.
4. Wayne SL, Rhyne RL, Garry PJ *et al.* (1990) Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. *J Gerontol* **45**, M45–M48.
5. High KP (2004) Infection as a cause of age-related morbidity and mortality. *Ageing Res Rev* **3**, 1–14.
6. Castle SC, Uyemura K, Fulop T *et al.* (2007) Host resistance and immune responses in advanced age. *Clin Geriatr Med* **23**, 463–479.
7. Pawelec G, Barnett Y, Forsey R *et al.* (2002) T cells and aging. *Front Biosci* **7**, d1056–d1083.
8. De la Fuente M, Hernanz A & Vallejo C (2005) The immune system in the oxidation stress conditions of aging and hypertension. Favorable effects of antioxidants and physical exercise. *Antioxid Redox Signal* **7**, 1356–1366.
9. Gruver AL, Hudson LL & Sempowski GD (2007) Immunosenescence of aging. *J Pathol* **211**, 144–156.
10. Aw D, Silva AB, Palmer DB (2007) Immunosenescence: emerging challenges for an ageing population. *Immunology* **120**, 435–446.
11. De la Fuente M, Hernanz A, Guayerbas N *et al.* (2008) Vitamin E ingestion improves several immune functions in elderly men and women. *Free Radic Res* **42**, 272–280.
12. Gomez CR, Nomellini V, Frunce DE *et al.* (2008) Innate immunity and aging. *Exp Gerontol* **43**, 718–728.
13. Alonso-Fernandez P, Puerto M, Maté I *et al.* (2008) Neutrophils of centenarians show function levels similar to those of adults. *J Am Geriatr Soc* **56**, 2244–2251.
14. Solana R, Pawelec G & Tarazona R (2006) Aging and innate immunity. *Immunity* **24**, 491–494.
15. De la Rosa O, Pawelec G, Peralbo E *et al.* (2006) Immunological biomarkers of ageing in man: changes in both innate and adaptive immunity are associated with health and longevity. *Biogerontology* **7**, 471–481.
16. De la Fuente M (2008) Role of neuroimmunomodulation in aging. *Neuroimmunomodulation* **15**, 213–223.
17. Viveros MP, Arranz L, Hernanz A *et al.* (2007) A model of premature ageing in mice based on altered stress-related behavioural response and immunosenescence. *Neuroimmunomodulation* **14**, 157–162.
18. Puerto M, Guayerbas N, Alvarez P *et al.* (2005) Modulation of neuropeptide Y and norepinephrine on several leucocyte functions in adult, old and very old mice. *J Neuroimmunol* **165**, 33–40.
19. Arranz L, Caamano J, Lord JM *et al.* (2010) Preserved immune functions and controlled leukocyte oxidative stress in naturally long-lived mice: possible role of nuclear factor-kappaB. *J Gerontol A Biol Sci Med Sci* **65**, 941–950.
20. Arranz L, Lord JM & De la Fuente M (2010) Preserved ex vivo inflammatory status and cytokine responses in naturally long-lived mice. *Age* (doi: 10.1007/s11357-010-9151-y).
21. Wrona D (2006) Neural-immune interactions: An integrative view of bidirectional relationship between the brain and immune systems. *J Neuroimmunol* **172**, 38–58.
22. Besedovsky HO & Del Rey A (2007) Physiology of psychoneuroimmunology: a personal view. *Brain Behav Immun* **21**, 34–44.
23. Arranz L, Guayerbas N & De la Fuente M (2007) Impairment of several immune functions in anxious women. *J Psychosom Res* **62**, 1–8.
24. Arranz L, De Vicente A, Muñoz M *et al.* (2009) Impaired immune function in a homeless population with stress-related disorders. *Neuroimmunomodulation* **16**, 251–260.
25. Franceschi C (2007) Inflammaging as a major characteristic of old people: can it be prevented or cured? *Nutr Rev* **65**, S173–S176.
26. Arranz L, Fernandez C, Rodriguez A *et al.* (2008) The glutathione precursor N-acetylcysteine improves immune function in postmenopausal women. *Free Radic Biol Med* **45**, 1252–1262.
27. De la Fuente M, Baeza I, Guayerbas N *et al.* (2004) Changes with aging in several leukocyte functions of male and female rats. *Biogerontology* **5**, 389–400.
28. Baeza I, De Castro NM, Gimenez-Llort L *et al.* (2010) Ovariectomy, a model of menopause in rodents, causes a premature aging of the nervous and immune systems. *J Neuroimmunol* **219**, 90–99.
29. Guayerbas N & De la Fuente M (2003) An impairment of phagocytic function linked to shorter life span in two strains of prematurely aging mice. *Dev Comp Immunol* **27**, 339–350.
30. Viña J, Sastre J, Pallardo FV *et al.* (2006) Role of mitochondrial oxidative stress to explain the different longevity between genders. Protective effect of estrogens. *Free Radic Res* **40**, 1359–1365.
31. Lamas O, Marti A & Martinez JA (2002) Obesity and immunocompetence. *Eur J Clin Nutr* **56**, 542–545.
32. Bastard JP, Maachi M, Lagathu C *et al.* (2006) Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw* **17**, 4–12.
33. De la Fuente M & De Castro NM (2010) Obesity as a model of premature immunosenescence. *Curr Immunol Rev* (In the Press).
34. Arranz L, Gimenez-Llort L, De Castro NM *et al.* (2009) Social isolation during old age worsens cognitive, behavioral and immune impairment. *Rev Esp Geriatr Gerontol* **44**, 137–142.
35. Droge W (2005) Oxidative stress and ageing: is ageing a cysteine deficiency syndrome? *Phil Trans R Soc B* **360**, 2355–2372.
36. Miquel J & Ecónomos AC (1979) Favorable effects of the antioxidants sodium and magnesium thiazolidin carboxylate on the vitality and life span of *Drosophila* and mice. *Exp Gerontol* **14**, 279–285.
37. Richie JP Jr, Mills BJ & Lang CA (1987) Correction of a glutathione deficiency in the aging mosquito increase its longevity. *Proc Soc Exp Biol Med* **184**, 113–114.
38. Lesourd B (2006) Nutritional factors and immunological ageing. *Proc Nutr Soc* **65**, 319–325.
39. De la Fuente M, Guayerbas N, Catalán MP *et al.* (2002) The amount of thiolic antioxidant ingestion needed to improve the

NS Proceedings of the Nutrition Society

immune functions is higher in aged than in adult mice. *Free Radic Res* **36**, 119–126.
40. Baeza I, De Castro NM, Alvarado C *et al.* (2007) Improvement of immune cell functions in aged mice treated for 5 weeks with soybean isoflavones. *Ann NY Acad Sci* **1100**, 497–504.
41. Alvarado C, Alvarez P, Puerto M *et al.* (2006) Dietary supplementation with antioxidants improves functions and decreases oxidative stress of leukocytes from prematurely aging mice. *Nutrition* **22**, 767–777.
42. Alvarado C, Alvarez P, Jimenez L *et al.* (2005) Improvement of leukocyte functions in young prematurely aging mice after a 5-week ingestion of a diet supplemented with biscuits enriched in antioxidants. *Antioxid Redox Signal* **7**, 1203–1210.
43. Alvarado C, Alvarez P, Jimenez L *et al.* (2006) Oxidative stress in leukocytes from young prematurely aging mice is reversed by supplementation with biscuits rich in antioxidants. *Dev Comp Immunol* **30**, 1168–1180.
44. Alvarez P, Alvarado C, Puerto M *et al.* (2006) Improvement of leukocyte functions in prematurely aging mice after five weeks of diet supplementation with polyphenol-rich cereals. *Nutrition* **22**, 913–921.
45. De la Fuente M, Ferrández MD, Del Rio M *et al.* (1998) Enhancement of leukocyte functions in aged mice supplemented with the antioxidant thioproline. *Mech Ageing Dev* **104**, 213–225.
46. Victor VM, Rocha M, Esplugues JV *et al.* (2005) Role of free radicals in sepsis: Antioxidant therapy. *Curr Pharm Des* **11**, 3141–3158.
47. Correa R, Blanco B, Del Rio M *et al.* (1999) Effect of a diet supplemented with thioproline on murine macrophage function in a model of premature ageing. *Biofactors* **10**, 195–200.
48. Puerto M, Guayerbas N, Victor VM *et al.* (2002) Effects of N-acetylcysteine on macrophage and lymphocyte functions in a mouse model of premature ageing. *Pharmacol Biochem Behav* **73**, 797–804.
49. Guayerbas N, Puerto M, Ferrandez MD *et al.* (2002) A diet supplemented with thiolic antioxidants improves leukocyte function in two strains of prematurely ageing mice. *Clin Exp Pharmacol Physiol* **29**, 1009–1014.
50. Guayerbas N, Puerto M, Alvarez P *et al.* (2004) Improvement of the macrophage functions in premature ageing mice by a diet supplemented with thiolic antioxidants. *Cell Mol Biol* **50**, OL677–OL681.

NS Proceedings of the Nutrition Society

https://doi.org/10.1017/S0029665110003848 Published online by Cambridge University Press