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ORIGINAL ARTICLE

Effect of diet supplementation with N-acetylcysteine on leucocyte functions in prematurely aging mice

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Summary
According to the free radical theory of aging, oxidative stress plays a key role in the senescent decline of the immune system, and therefore antioxidants may provide an effective therapy in order to protect the immune function against oxidative damage. Accordingly, the purpose of this study was to investigate the favourable effect of a diet supplemented with a glutathione precursor – namely the antioxidant N-acetylcysteine (NAC, 0.1% w/w) – on several functions of the leucocytes from prematurely-aging mice (PAM). In adult PAM, the immune functions studied showed values similar to those of older mice, and worse than those found in adult non prematurely-aging mice (NPAM). A short-term ingestion (4 weeks) of NAC, although unable to influence the NK activity, the IL-2 release or the glutathione content of the peritoneal leucocytes of PAM, resulted in an improvement of other very important leucocyte functions such as the lymphoproliferative response to the mitogen Con A, and IL-1β and TNFα release.

Keywords: antioxidant – glutathione – leucocytes – lymphoproliferation – N-acetylcysteine

INTRODUCTION

According to the most widely accepted theory proposed to explain the process of aging, the is process is linked to the progressive oxidation damage to biomolecules, as the result of chronic oxidative stress, i.e. to an imbalance between antioxidant defences and free radical production. In the immune system, the functional state of which is a very good marker of health and longevity (Wayne et al. 1990, Aspinall 2000), the cells are specially involved with free radical generation in order to carry out their functions, such as microbicidal and cytotoxic activities. Further, the antioxidant/ oxidant balance is an important determinant of immune cell function (De la Fuente 2002). Antioxidant deficiencies are usually associated with an impaired immune response – particularly cell-mediated immunity – which leads to frequent severe infections resulting in increased mortality (Chandra 1999, Knight 2000). Specially, reduced glutathione (GSH) levels are considered an important and efficient antioxidant mechanism that maintains cells in a reduced condition and protects the organs and tissues against oxidative damage (Dröge 2002). An age-related decrease in intracellular glutathione has been observed in various cells and tissues, including the immune

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system (Franklin et al. 1990, Goldstone and Hunt 1997, Hernanz et al. 2000) and, accordingly, diet supplementation with thiolic compounds, which act as glutathione precursors, such as N-acetylcysteine (NAC), could have favourable effects, replenishing the glutathione levels and thus preventing the age-related decline of the immune system.

In agreement with the above, we have carried out a study in a prematurely aging mouse model. Mice which take longer to complete the exploration of the first arm of a T-shaped maze (PAM, prematurely aging mice) show, at the same chronological age as those which quickly explore the maze (NPAM, non prematurely aging mice), values of several nervous (behavioural and monoaminergic parameters) and immune system functions (Guayerbas et al. 2000, 2002a, 2002b, Viveros et al. 2001, Guayerbas and De la Fuente 2003, De la Fuente et al. 2003b) similar to those found in older animals of the same strain, which results in a shorter life span. Because PAM exhibit worse immune functions than NPAM of the same chronological age, we have observed in a previous study that a diet supplemented with 0,1% (w/w) of NAC has favourable effects in PAM on several functions of macrophages and lymphocytes, such as adherence to substrate, chemotaxis, phagocytosis and intracellular ROS production, (Puerto et al. 2002). The aim of the present work is to find out if that improvement also occurs in other important functions of leucocytes of these animals. Thus, we have investigated the lymphoproliferative response, NK activity and production of several cytokines such as, IL-1β, TNFα and IL-2 have been investigated.

MATERIALS AND METHODS

Animals

Female ICR (CD-1) mice (Harlan Ibérica, Spain), which were 24 weeks old on arrival at our laboratory, were used. The mice were specific pathogen free, as tested by Harlan according to FELASA recommendations. The animals were randomly divided into groups of 5, each group being housed in a polyurethane box, at a constant temperature (22±2ºC) under sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan France) on a 12/12 h reversed light/dark cycle. All animals were fed Sander Mus (A. 04 diet from Panlab L.S Barcelona, Spain) pellets and water *ad libitum.* The diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals. The mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC.

Experimental procedure

At 25 weeks of age, the spontaneous exploratory behavior of each mouse was tested in a T-shaped maze (with arms 50 cm in length). The test is performed holding the mouse from the tip of the tail and placing it inside the "vertical" arm of the maze with its head facing the end wall. The performance is evaluated by determining with a chronometer the time elapsed until the animal crosses with both hind legs the intersection of the three arms. This test was performed four times, once a week, in order to sort out the NPAM (non prematurely aging mice), which complete the exploration of the first arm of the maze in 20 seconds or less, from the PAM (prematurely aging mice), which always require over 20 seconds on all four occasions. Animals showing an intermediate response in the T maze were removed from the study. Thus, we had two groups of animals, one group contained the control population (NPAM) and the other the PAM population. Then, each group was subdivided into a control population (C) and a treated with antioxidants (A) population, at 29 weeks of age. Therefore, we had the following groups: NPAMC, NPAMA, PAMC and PAMA. The treated animals received a diet supplemented with 0.1% w/w of N-acetylcysteine (NAC) (Sigma, St. Louis, USA) for four weeks.

Collection of peritoneal leucocytes

At 33 weeks of age, peritoneal suspensions were obtained without sacrificing the mice. Mice were held by the cervical skin, the abdomen was cleansed with 70% ethanol, and 3ml of sterile Hank's solution were injected intraperitoneally. After massaging the abdomen, 80% of the injected volume was recovered. Peritoneal leucocytes were counted and then adjusted to $1x10^6$cells/ml of medium. The cellular viability, determined in each experiment using the trypan-blue exclusion test, was in all cases higher than 95%.To determine the number of the different kind of peritoneal leucocytes, samples were analyzed by flow cytometry (FAC Scan, Becton Dickinson, USA) using Cell-Quest (Becton Dickinson) software on the basis of the different size and structural complexity of the lymphocytes, macrophages and NK cells.

Assay of leucocyte functions

The proliferation of leucocytes induced by Con A mitogen, was determined in 72h cultures. Aliquots (200µl) of peritoneal suspensions, adjusted to 1×10^6 leucocytes/ml of medium (RPMI 1640, Gibco, Pasley, Scotland, UK) supplemented with 10% foetal calf serum (FCS) (Gibco), previously inactivated by heat (30min at 56ºC) and with gentamicin (10mg/ml, Gibco), were seeded in 96-well flat-bottomed microtiter plates and incubated

in the presence of Concanavaline A (Con A, 1μg/ml, Sigma) for 48h at 37ºC in an atmosphere of 5% CO_2. Then 5μl of ^{3}H-thymidine (5mCi) were added to each well and after 8 h the cells were harvested in a semiautomatic microharvester and thymidine uptake was measured in a beta counter (LKB) for 1min. The results were expressed as ^{3}H-thymidine uptake (c.p.m.).

The NK activity of the leucocytes from the peritoneum was determined following a method previously described (Ferrández et al. 1999). An enzymatic colorimetric assay (Cytotox 96 TM Promega, Boeringher Ingelheim) was used for cytolysis measurements of target cells (YAC-1 cells from a murine lymphoma), which is based on the determination of lactate dehydrogenase (LDH) enzymatic activity, using tetrazolium salts. The cells were maintained in a complete medium (RPMI-1640 plus 10% FCS). Target cells (YAC-1 cells) were seeded in 96-well U bottom culture plates, at 10^4cells/well, in 1640 RPMI without phenol red. Effector cells (peritoneal leucocytes) were added at 10^5cells/well, being the effect/target rate 10/1. The plates were centrifuged at 250g for 4min to facilitate cell contacts and then they were incubated for 4 h at 37ºC. After incubation, LDH activity was measured in 50 ml/well of the supernatants by addition of the enzyme substrate and absorbance recording at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. To determine the percentage of lysis in the target cells, the following equation was used: % lysis = ((E-ES-TS)/(M-ES-TS)) × 100, where E = mean of absorbances in the presence of effector cells, ES = mean of absorbances of effector cells incubated alone, TS = mean of absorbances in target cells incubated with medium alone, and M = mean of maximum absorbances after incubating target cells with lysis solution.

The concentration of interleukin-2 (IL-2) was determined in culture supernatants of leucocytes following a method previously described by us (Medina et al., 2000). After 48h of incubation with the mitogen Con A the supernatants were collected and the IL-2 measured using an ELISA kit (R&D System, Minneapolis, USA). The results were expressed as pg/ml.

The mouse tumour necrosis factor (TNFα) and IL-1β release were measured in the supernatants of cultures of peritoneal leucocytes as we have previously described (Víctor et al. 1999). Cell suspensions were incubated with Hank´s solution at a final concentration of 2×10^5 cells/200μl/well in 96 well plates for 60min to allow leucocytes to form a monolayer, and after this time, lypopolysaccharide (LPS, *E. coli*, 055:B5, Sigma, 10 μg/ml) was added. After 24h of incubation the supernatants were collected and the concentration of cytokines was measured using an ELISA kit (Endogen, Woburn, USA) and the results were expressed as pg/ml.

The determination of reduced glutathione (GSH) was carried out in aliquots of 300μl using a colorimetric assay Bioxytech GSH 400 (OXIS International, Inc.). The GSH-400 method is based on a chemical reaction which proceeds in two steps. The first step leads to the formation of substitution products (thioethers) between a patented reagent, R1 (4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate), and all mercaptans (RSH) which are present in the sample (300μl supernatant + 600μl of PBS + 50μl RI). The second step is a β-elimination reaction which takes place under alkaline conditions (+50μl R2). This reaction is mediated by reagent R2 (30% NaOH) which especifically transforms the substitution product (thioeter) obtained with GSH into a chromophoric thione which has a maximal absorbance wavelength at 400nm. The samples were incubated for 10min in the dark at 25ºC and afterwards measured at 400nm in a spectrophotometer.

Statistical analysis

The data are expressed as the mean ± SD. The normality of the samples was confirmed by the Kolmogorov-Smirnov test and the homogeneity of variances by the Levene test. The two-way analysis of variance (ANOVA) and the Tukey test were used for the comparison of parametric samples at the significance level $2\alpha=0.05$.

RESULTS

Peritoneal leucocyte subsets

The percentages of peritoneal lymphocytes, macrophages and NK cells, with respect to the total peritoneal leucocyte population, in NPAMC, were: 68.1±4.7, 13.28±3.0 and 18.33±5.6, respectively. No statistically significant differences were found in the other experimental groups (PAMC, NPAMA and PAMA) as compared to the NPAMC group.

Immune function

The results of the lymphoproliferative response to the mitogen Con A are shown in Fig. 1A. This capacity was lower in the PAMC group than in the NPAMC group (statistically significant). The supplementation of the diet with 0.1% of the antioxidant NAC increased significantly (statistically significant) the proliferation of leucocytes of PAM (PAMA). As regards NK activity, no significant differences were found between NPAMC and PAMC, and the antioxidant ingestion did not have any effect (44±12 and

47±13 for NPAMC and PAMC respectively; 46±13 and 44±8 for NPAMA and PAMA respectively). As regards cytokine production (Table. 1), statistical differences between NPAMC and PAMC were observed in IL-2 and IL-1β release, with the PAMC showing the lower levels (statistically significant). Dietary supplementation with NAC increased the IL-1β levels only in the PAMA.

Table 1. **Effect of NAC treatment on cytokines release and GSH levels of peritoneal leucocytes.**

	NPAMC	PAMC	NPAMA	PAMA
IL-2 (pg/ml)	367±49	182±67*	383±32	191±39
IL-1β (pg/ml)	295±14	126±27*	233±41	214±41•
GSH content (μmols/10^6 cells)	109.20±10	94.7±6.3*	103±6	98.4±7.4

Each date represents the mean±S.D. of 8 values corresponding to 8 animals, each value being the mean of duplicate assays. * statistically significant with respect to the corresponding values in the NPAMC. • statistically significant with respect to the corresponding values in the control animals. NPAM: non-prematurely aging mice; NPAMC: NPAM, controls; NPAMA: NPAM treated with 0,1% of NAC; PAM: prematurely aging mice; PAMC: PAM, controls; PAMA: PAM treated with 0.1% of NAC.

Oxidative stress

Regarding the proinflammatory cytokine, namely the TNFα (Fig. 1B), NAC supplementation decreased (statistically significant) its levels in both groups, i.e., PAM and NPAM. The GSH levels in peritoneal leucocytes (Table. 1) were lower in the PAMC than in the NPAMC (statistically significant), but the treatment with 0.1% NAC did not increase these levels in the cells of the peritoneum.

DISCUSSION

Limphoproliferation

In the present study we have observed the beneficial effects of dietary supplementation with 0.1% of NAC for four weeks in mice, mainly in PAM. These animals showed a decrease in lymphoproliferation in response to Con A as well as in the IL-1β and IL-2 release, which are typical changes that accompany the aging process, previously reported in PAM (Guayerbas et al. 2002b). An increased level of inflammatory cytokines, such as TNFα is the other age-related change (Bruunsgaard et al, 2001) which has also been reported in PAM (Guayerbas et al. 2002b). These observations provide evidence of the key role that the oxidation and/or inflammation state plays in the aging process. After NAC supplementation, an improvement of the lymphoproliferative response to Con A was found in the PAM, which reached the levels of the NPAM group. Similar results were shown by Viora et al. (2001), who found a significant down-regulation of proliferative response to mitogens caused by treatment with oxidized low-density lipoproteins whereas after the NAC exposure the above function showed values comparable to the controls.

Cytokines and NK activity

The favourable effects of NAC were also observed in the modulation of the cytokines studied such as IL-1β, IL-2 and TNFα. Thus, the dietary administration of NAC in the PAM group increased the IL-1β levels, which play an important regulatory role in T-cell activation. Moreover, the protective effect of NAC was shown by a significant reduction of the TNFα levels in both groups. In a model of lethal endotoxic shock, which showed an increase of TNFα release by peritoneal leucocytes, the administration of NAC, at 30 min after the injection of endotoxin, also decreased these high levels of TNFα (Víctor et al. 2003). Moreover, since the TNFα is known to be regulated by the redox-responsive transcription factor, NF-kB in this model of endotoxic shock, NAC prevented the inflammatory state, decreasing the NF-kB transcriptional activity in peritoneal lymphocytes and macrophages (Víctor et al. 2004).

In the same way, Sekhon et al. (2003) have demonstrated that pretreatment with NAC inhibits the expression of TNFα providing protection from injury in a rat model of ischemia. However, the NK activity and the IL-2 release were not influenced by the NAC treatment, suggesting that a longer-term ingestion may be needed to improve these functions.

Glutathione levels

Another important finding of this study was that the content of GSH was significantly lower in the peritoneal leucocytes of PAMC than in those of NPAMC, which could be responsible for the lower lymphoproliferative response of the cells from PAM as compared to those from NPAM. In agreement with the above, several authors have pointed out the important role of GSH in the lymphoproliferative capacity (Viora et al. 2001), which is very closely linked to the maintenance of the redox balance (Pieri et al. 1992). However, a four-week administration of NAC did not result in an increase of GSH content. This result could be explained by the short period of the treatment and/or the low antioxidant concentration. In fact,

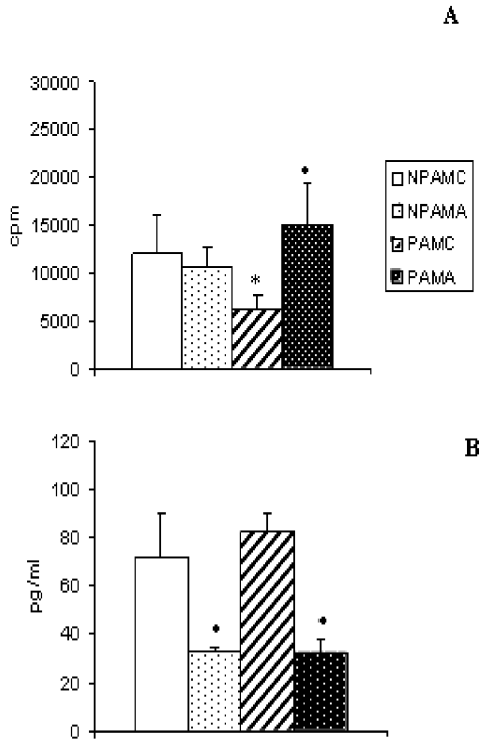

Fig. 1. **Lymproliferation and TNFα release.** Lymphoproliferative response to the mitogen Con A (1µg/ml) (1A) and TNFα release (pg/ml) (1B) of peritoneal leucocytes. Each column represents the mean±S.D. of 8 values corresponding to 8 animals, each value being the mean of triplicate assays.

•statistically significant with respect to the corresponding values in the control animals
*statistically significant with respect to the corresponding values in the NPAMC
NPAM: non-prematurely aging mice
NPAMC: NPAM controls
NPAMA: NPAM treated with 0.1% of NAC
PAM: prematurely aging mice
PAMC: PAM controls
PAMA: PAM treated with 0.1% of NAC

recent studies of our laboratory have demonstrated that supplementation for five weeks of 0.1% of NAC + 0.1% of TP (w/w) enhances the GSH levels of peritoneal leucocytes (De la Fuente et al. 2003a).

In summary, the immune response improvement observed after NAC diet supplementation could be due to a direct immunostimulatory action of this thiolic antioxidant. A similar conclusion was obtained by Guevara et al. (2000) in an experimental infection model with *Trypanosoma cruzi*. They observed that NAC up-regulated the proliferation of the spleen cells of infected mice and modified the pattern of subpopulations of lymphocytes linked to the reduction of parasitemia levels. Moreover, previous studies from our laboratory have demonstrated the immunomodulatory role of NAC on the phagocytic capacity of peritoneal macrophages of PAM, which improved after four weeks of NAC (0.1% w/w) ingestion (Puerto et al. 2002). Because of the low toxicity of NAC, the functions which failed to show an effect, such as NK activity and IL-2 release, could also be stimulated by higher concentrations or a longer ingestion period of this antioxidant.

CONCLUSIONS

The results of the present work provide new insights into the key role played by NAC as a modulator of the immune system functions especially in subjects biologically older (PAM), and suggest that thiolic antioxidants could be useful in order to counteract age-related decline of the immune system.

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