ELSEVIER

Pharmacology, Biochemistry and Behavior 73 (2002) 797–804

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

www.elsevier.com/locate/pharmbiochembeh

Effects of *N*-acetylcysteine on macrophage and lymphocyte functions in a mouse model of premature ageing

Marta Puerto, Noelia Guayerbas, Víctor M. Víctor, Mónica De la Fuente*

Departmento de Fisiología Animal, Facultad de Biología, Universidad Complutense, José Antonio Novais s/n, E-28040 Madrid, Spain

Received 6 February 2002; received in revised form 27 May 2002; accepted 28 May 2002

Abstract

In previous studies, we have observed that mice of the same strain and age show striking interindividual differences in behavior when exposed to a T-maze test. The animals that take longer to explore a T-shaped maze ("slow" animals) show high levels of emotionality/anxiety in other standard behavioral tests, prematurely aged immune functions, and a shorter life span, in comparison to "fast" mice. In these slow mice, which are a model of premature immunosenescence, the immune functions were improved after the ingestion of the thiolic antioxidant thioproline in the diet. In the present work, we studied the effects in vivo (0.1% w/w, for 4 weeks) and in vitro (0.001, 0.01, 0.1, 1, and 2.5 mM) of the thiolic antioxidant *N*-acetylcysteine (NAC) on different functions of peritoneal macrophages and lymphocytes from slow and fast adult Swiss mice. The results showed an improvement of all the functions studied, namely adherence to substrate, directed migration or chemotaxis, phagocytosis, and reactive oxygen species (ROS) production, after in vivo and in vitro treatment with NAC. The effect of this antioxidant was stronger in the cells from the slow than in those from the fast mice.
© 2002 Elsevier Science Inc. All rights reserved.

Keywords: Antioxidants; *N*-Acetylcysteine; Macrophage; Lymphocyte; Ageing

1. Introduction

Ageing is associated with a decline of many physiological functions, including those of the nervous and the immune system, as well as the relationship of these two systems (Burgess et al., 1999; Fabris, 1991; Goya et al., 1999), which leads to a loss of homeostasis. Ageing of the immune system involves many changes in all aspects of the immune response (Ginaldi et al., 1999; McArthur, 1998; Pawelec, 1999). T-lymphocyte functions are especially influenced by ageing (Ginaldi et al., 1999; Pawelec, 1999; Pahlavani and Richardson, 1996), and the changes occurring in these cells have been associated with alterations in the intracellular signaling pathways (Hirokawa, 1999). However, for some authors, phagocytic cells do not change throughout life while others have observed a senescent decrease or increase in their functions (Ginaldi et al., 1999; McArthur, 1998; De la Fuente et al., 2000; Ortega et al., 1993, 2000; Solana and Pawelec, 1998).

On the other hand, ageing does not affect all individuals in the same way, i.e., interindividual differences in the rate of ageing suggest that chronological and biological age do not necessarily coincide (Dellu et al., 1994). Thus, several studies on mice have related the response in behavioral tests to biological age (Dellu et al., 1994) and to life span (Gilad and Gilad, 1995). The immune system has been proposed as a marker of biological age and life span (Wayne et al., 1990) since a suboptimal immune function may significantly contribute to morbidity and mortality in the elderly (Ginaldi et al., 1999).

In agreement with the above, previous work from our laboratory showed that interindividual differences in life span among members of Swiss outbred mouse populations may be related to their behavior in a simple T-maze test, with most mice that quickly explored the maze (fast mice) reaching a longer life span than mice that took longer to accomplish this task (Guayerbas et al., 2000). Furthermore, a relation between T-maze performance and the immune status of the animals has been shown (Correa et al., 1999a; Viveros et al., 2001; Guayerbas et al., 2002, in press). Thus, fast mice present a better immune system than slow mice, which show an immune system similar to that of aged

* Corresponding author. Tel.: +34-91-394-4989; fax: +34-91-394-4935.

E-mail address: mondelaf@bio.ucm.es (M. De la Fuente).

0091-3057/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0091-3057(02)00902-4

animals. Therefore, slow mice can be considered a model of premature ageing and immunosenescence.

Immune cells, particularly phagocytes, produce oxygen radicals needed to carry out their functions. However, they can become a source of tissue damage if their production is not controlled by the antioxidant defenses of the cell, thus resulting in oxidative stress (Knight, 2000). For many years, it has been well known that reactive oxygen species (ROS) may contribute to ageing of the immune system. Moreover, a great longevity may be associated with an optimal antioxidant protection (Sahnoun et al., 1998). Accordingly, in vitro and in vivo improvement of the functions of immune cells from adult and old animals by antioxidants has been shown by us and other authors (Meydani et al., 1998; Blanco et al., 1999; Del Río et al., 1998; Ferrández et al., 1999).

N-Acetylcysteine (NAC), a thiolic antioxidant, has been studied by many authors because of its wide range of effects at all cellular levels. NAC has inhibitory effects on apoptosis (Heussler et al., 1999), proinflammatory cytokine production (Gosset et al., 1999), carcinogenic action of some compounds (Chatterjee and Deb, 1999), and metastases (De la Flora et al., 1995). In previous studies, our group has observed a stimulatory effect of NAC, in vitro, on several functions of murine macrophages (Del Río et al., 1998) and NK activity (Ferrández et al., 1999) as well as a modulatory effect on macrophage and lymphocyte function in a model of endotoxic shock (De la Fuente and Víctor, 2000; Víctor and De la Fuente, 2002; Víctor et al., 1999).

Since in the model of immunosenescence commented above the supplementation of the diet with thioproline improved immune function (Correa et al., 1999a), the aim of the present work was to test the effect of NAC, in vivo and in vitro, on several functions of peritoneal macrophages and lymphocytes from slow and fast female Swiss mice.

2. Materials and methods

2.1. Animals and experimental conditions

We have used female outbred Swiss (Harlan Interfauna Ibérica, Barcelona, Spain) adult mice (*Mus musculus*), which were 22 weeks old on arrival at our laboratory. The animals used did not show any sign of malignancy or other pathological processes and they were housed in groups of seven to nine individuals per cage and maintained at a constant temperature of 21 ± 1 °C and in a reverse 12:12-h dark–light cycle (lights on 20:00 h) with free access to food (A04 diet from Panlab L.S., Barcelona, Spain) and water. The animals were marked for their individual follow-up. Five days after their arrival to the laboratory, the mice were tested individually in a T-shaped maze. The performance of the test was 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 for 4 weeks, once a week, in order to sort out the "fast" mice (which completed the exploration of the first arm of the maze in 20 s or less) from the "slow" mice (which required over 20 s). Then, animals were distributed in two groups. One group contained the fast population and the other the slow population with a fast/slow ratio of 100/0 and 0/100, respectively.

For the in vivo study, fast and slow mice were divided into control and treated groups, with eight animals per group. The treated groups received a diet supplemented with 0.1% w/w of NAC during 4 weeks.

Mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC.

2.2. Reagents

NAC was purchased from Sigma (St. Louis, MO, USA) and was used at the following concentrations: 0.001, 0.01, 0.1, 1, and 2.5 mM dissolved in Hank's solution. Formulated peptide (fMet–Leu–Phe, fMLP), latex beads (1.09 μm), and phorbol myristate acetate (PMA) were also obtained from Sigma. Dichlorodihydrofluorescein diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR, USA). Hank's solution was prepared as follows: 5.5 mM glucose, 1 mM $MgCl_2$, 136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.8 mM MgH_2PO_4, 0.5 mM KH_2PO_4, 0.4 mM Na_2HPO_4, and 4 mM $NaHCO_3$, adjusted to pH 7.4.

2.3. Collection of immune cells

For both in vitro and in vivo studies, cells were obtained following an intraperitoneal injection of Hank's medium (3 ml) without sacrifice of the animals. The resting peritoneal leukocytes (macrophages and lymphocytes) were collected, identified, and quantified by their morphology and cytometric analysis (the peritoneal suspension contained 45% of macrophages and 55% of lymphocytes). Cellular suspensions were adjusted to 5×10^5 or 1×10^6 macrophages/ml Hank's solution. Cellular viability, routinely measured before and after each experiment by the trypan blue exclusion test, was higher than 95% in all experiments. All incubations were performed at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. Assay of macrophage and lymphocyte functions

Functions studied for macrophages were substrate adherence, directed migration or chemotaxis, phagocytosis of inert particles, and ROS production. For lymphocytes, the same functions were studied with the exception of phagocytosis.

The quantification of substrate adherence capacity was carried out following a method previously described (De la Fuente et al., 1991; Ortega et al., 2000). Aliquots of 200 μl of peritoneal suspension (adjusted to 5×10^5 macrophages/ml Hank's medium) were placed in Eppendorf tubes and

20 μl of the antioxidant, at different concentrations, or 20 μl of Hank's medium (controls) were added (for the in vitro study). At 10, 20, or 30 min of incubation, aliquots of 10 μl from each sample were removed after gently shaking to resuspend the sedimented cells, and the number of non-adhered macrophages and lymphocytes was determined using a Neubauer chamber and optical microscopy. The adherence indexes (AI) were calculated according to the following equation: AI =(1 − cells/ml supernatants/cells/ml original sample) × 100.

Chemotaxis was evaluated by a modification (De la Fuente et al., 1991; Ortega et al., 2000) of Boyden's technique (1962), which basically consists in the use of chambers with two compartments separated by a transparent filter of 3 μm of pore diameter (Millipore, Madrid, Spain). Aliquots of 300 μl of peritoneal suspensions (adjusted to 5×10^5 macrophages/ml Hank's medium) were deposited in the upper compartment with 30 μl of the antioxidant, at the different concentrations for the in vitro study, or Hank's medium in the controls. In the lower compartment, the chemoattractant fMLP, at the concentration of 10^{-8} M, was deposited for determination of chemotaxis. The chambers were incubated for 3 h, the filters fixed and stained, and macrophages and lymphocyctes were counted using optical microscopy (immersion objective). Chemotaxis was expressed as number of macrophages or lymphocytes in one-third of the lower face of the filter.

Phagocytosis of inert particles (latex beads diluted to 1% in phosphate-buffered saline, PBS) was carried out, following the method previously described (De la Fuente et al., 1991; Ortega et al., 2000), incubating aliquots of 200 μl of the peritoneal suspension (adjusted to 5×10^5 macrophages/ml Hank's medium) in migratory inhibitory factor (MIF) plates (Kartell, Noviglio, Italy) for 30 min. The adhered monolayer was washed with PBS at 37 °C, resuspended in 200 μl of Hank's medium, and incubated with 20 μl of latex, as well as with 20 μl of the different concentrations of the antioxidant or Hank's medium (for the in vitro study). After 30 min of incubation, the plates were washed with PBS, fixed, and stained, and the phagocytosis was expressed as the number of latex beads ingested per 100 macrophages, which was assessed by optical microscopy. The number of ingesting macrophages per 100 macrophages was also determined.

The ROS production was measured by flow cytometry. DCF-DA was used as a probe since it is oxidized in the cytoplasm by ROS to 2′,7′-dichlorofluorescein (DCF), which is a highly fluorescent compound. Aliquots of 200 μl of the peritoneal suspension (adjusted to 10^6 macrophages/ml) were centrifuged during 10 min at 1500 rpm and 4 °C. Supernatants were discarded and pellets were resuspended in 200 μl of Buffer A (Hank's solution without Ca^{2+} and Mg^{2+} and with EGTA 1 mM). Samples were incubated with 2 μl of DCF-DA (0.5 mM) for 15 min at 37 °C. After incubation, 40 μl of PMA and 40 μl of Buffer A were added to stimulated and control samples, respectively. For the in vitro study, 40 μl of NAC at a concentration of 1 mM was also added (final concentration 0.1 mM). Samples were incubated during 15 min at 37 °C and then were analysed in the flow cytometer. Macrophages and lymphocytes were analysed separately by differences in forward scatter and size scatter. Results were expressed as fluorescence units.

2.5. Statistical analysis

All values are expressed as the mean ± S.D. of the number of animals used for assay of the different functions, as indicated in the corresponding tables and figures. The normality of the samples was checked by the Kolmogorov–Smirnov test. The data were statistically evaluated by the Mann–Whitney U test for unpaired observations of non-parametric data as well as by the Wilcoxon's test for paired comparison between samples with and without NAC, with a minimum significance level of $P < .05$.

3. Results

3.1. Effect of NAC on macrophage and lymphocyte functions from untreated slow and fast mice

3.1.1. Macrophage functions

The early adherence (at 10 min of incubation) (Table 1) was higher in the slow than in the fast group and was significantly stimulated by NAC in the fast mice at the higher concentrations (1 and 2.5 mM). At 20 min of incubation, the AI was higher for the macrophages from slow mice and there was no effect of NAC in any group. There were no differences in adherence capacity between groups at 30 min of incubation and the antioxidant had no effect at this time.

In regard to chemotaxis capacity (Fig. 1A), the slow group showed a significantly lower index than the fast group, and NAC increased this function, especially in the slow mice. The phagocytic capacity, measured as the number of particles phagocytosed per 100 macrophages, is shown in Fig. 1B. The phagocytosis index was decreased in the slow mice in comparison to the fast group and was increased by the antioxidant at all concentrations used. In addition, the percentage of phagocytosing cells (Table 2) was lower in the slow mice and increased with the higher concentrations of NAC (1 and 2.5 mM).

The ROS production (Table 3) was similar in both groups in the absence of stimulation. Macrophages stimulated with PMA showed an increased ROS production in the fast and in the slow mice, the values being smaller in the slow mice with respect to the fast mice. Macrophages incubated with NAC (0.1 mM) presented similar levels of ROS to those found in the absence of stimulation, although the ROS level was lower in the slow mice. However, macrophages incubated with both PMA and NAC showed a decreased ROS

Table 1
Effect of in vitro NAC on AI of macrophages and lymphocytes from fast and slow Swiss mice

Cell	B	T	NAC concentrations (mM)					
			0	0.001	0.01	0.1	1	2.5
Macrophages	fast	10	29 ± 5	31 ± 7	31 ± 8	31 ± 8	49 ± 8†	39 ± 6†
		20	46 ± 9	51 ± 8	45 ± 10	50 ± 10	44 ± 12	51 ± 8
		30	61 ± 13	64 ± 7	63 ± 8	64 ± 11	63 ± 10	65 ± 13
	slow	10	38 ± 7**	37 ± 7	35 ± 9	36 ± 7	37 ± 7	36 ± 7
		20	57 ± 10*	59 ± 12	49 ± 10	51 ± 10	57 ± 10	56 ± 11
		30	66 ± 12	63 ± 14	59 ± 13	59 ± 9	64 ± 11	61 ± 11
Lymphocytes	fast	10	28 ± 7	36 ± 5†	41 ± 5†	40 ± 6†	42 ± 7†	46 ± 9†
		20	43 ± 9	52 ± 9	54 ± 9	53 ± 9	54 ± 10	55 ± 7
		30	48 ± 9	50 ± 9	49 ± 8	49 ± 9	47 ± 10	51 ± 8
	slow	10	38 ± 6***	35 ± 7	37 ± 6	36 ± 7	36 ± 6	34 ± 7
		20	54 ± 9**	45 ± 13	47 ± 9	46 ± 7†	50 ± 7	50 ± 7
		30	56 ± 9	44 ± 10†	42 ± 6†	47 ± 11	57 ± 6	54 ± 8

Each value is the mean ± S.D. of eight values corresponding to eight independent experiments. B = behavior in T-maze; T = time of incubation (min).
* $P<.05$ with respect to the fast group.
** $P<.01$ with respect to the fast group.
*** $P<.001$ with respect to the fast group.
† $P<.05$ with respect to the corresponding control.

production with respect to PMA-stimulated cells in the slow as well as in the fast animals with smaller values for the slow group with respect to the fast group.

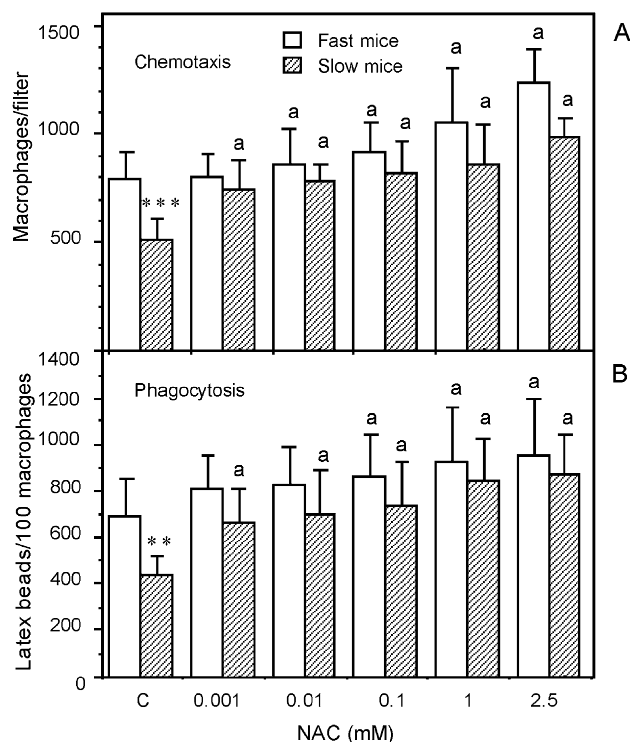

Fig. 1. Chemotaxis and phagocytosis of latex beads by peritoneal macrophages incubated with NAC (0.001, 0.01, 0.1, 1, and 2.5 mM). Each column represents the mean ± S.D. of 10 values (macrophages/filter and latex beads/100 macrophages) corresponding to 10 independent experiments. ** $P<.01$ with respect to fast group; [a]$P<.05$ with respect to slow and fast controls (C).

3.1.2. Lymphocyte functions

Adherence at 10-min incubation of peritoneal lymphocytes (Table 1) was higher in the slow mice than in the fast group and was significantly increased by NAC at all concentrations used in the fast mice, whereas no effect was observed in the slow group. At 20 min of incubation, adherence of lymphocytes from the slow mice was again higher. NAC had a stimulatory effect on the fast group and decreased adherence in slow mice only at a concentration of 0.1 mM. No differences were observed in the AI between groups after 30 min of incubation. The antioxidant decreased this capacity in the slow animals only at the lower concentrations.

With regard to chemotaxis capacity (Fig. 2), it was significantly lower in the slow group in comparison with the fast animals. The antioxidant increased this capacity at all concentrations used in the slow mice and only at higher concentrations (0.1, 1, and 2.5 mM) in the fast group.

The ROS production (Table 4) was similar in nonstimulated lymphocytes from slow and fast mice. After stimulation with PMA, lymphocytes from fast and slow animals increased their production of ROS although this increase

Table 2
Effect of in vitro NAC on the percentage of phagocytosing macrophages from fast and slow Swiss mice

	NAC concentrations (mM)					
	0	0.001	0.01	0.1	1	2.5
Fast mice	62 ± 11	56 ± 14	56 ± 16	59 ± 15	64 ± 12	63 ± 13
Slow mice	46 ± 9**	48 ± 10	54 ± 7	55 ± 7	56 ± 8†	58 ± 8†

Each value is the mean ± S.D. of eight values corresponding to eight independent experiments.
** $P<.01$ with respect to the fast group.
† $P<.05$ with respect to the corresponding control.

Table 3
ROS production (fluorescence units) by peritoneal macrophages incubated with NAC

	Fast mice	Slow mice
DCF	100 ± 17	88 ± 21
DCF + PMA (50 ng/ml)	151 ± 30###	113 ± 23###, * *
DCF + NAC (0.1 mM)	107 ± 22	83 ± 20 *
DCF + PMA + NAC	113 ± 27††	87 ± 23†††, *

Each value is the mean ± S.D. of eight values (fluorescence units) corresponding to eight independent experiments.
* $P<.05$ with respect to the fast group.
* * $P<.01$ with respect to the fast group.
†† $P<.01$ with respect to the stimulated samples (PMA).
††† $P<.001$ with respect to the stimulated samples (PMA).
$P<.001$ with respect to the corresponding control (DCF).

was higher in the fast group. Lymphocytes incubated with NAC (0.1 mM) showed similar levels of ROS production to those in the absence of stimulation in the slow group but decreased their ROS production in the fast animals. Lymphocytes incubated with both PMA and NAC presented a decrease in the levels of ROS with respect to PMA-stimulated cells in the fast animals.

3.2. Effect of in vivo treatment with NAC on macrophage and lymphocyte functions from slow and fast mice

3.2.1. Macrophage functions

The different functions of macrophages are shown in Table 5. With regard to early (10-min) adherence (data not shown), no difference was observed between the slow (42 ± 6) and the fast control mice (38 ± 6) and this adherence was decreased ($P<.01$) by NAC in the slow animals

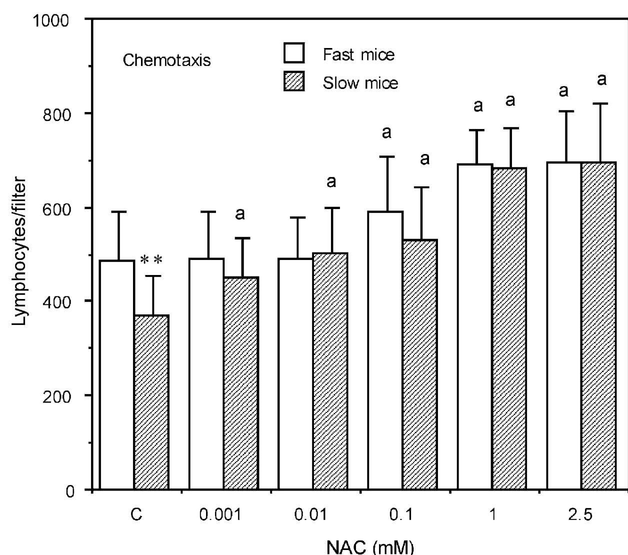

Fig. 2. Chemotaxis of peritoneal lymphocytes incubated with NAC (0.001, 0.01, 0.1, 1, and 2.5 mM). Each value represents the mean ± S.D. of 10 values (lymphocytes/filter) corresponding to 10 independent experiments. * * $P<.01$ with respect to fast group; [a]$P<.05$ with respect to slow and fast controls (C).

Table 4
ROS production (fluorescence units) by peritoneal lymphocytes incubated with NAC

	Fast mice	Slow mice
DCF	59 ± 13	49 ± 9
DCF + PMA (50 ng/ml)	81 ± 15###	58 ± 13#, * *
DCF + NAC (0.1 mM)	44 ± 9###	46 ± 11
DCF + PMA + NAC	54 ± 12†††	50 ± 11

Each value is the mean ± S.D. of eight values (fluorescence units) corresponding to eight independent experiments.
* * $P<.01$ with respect to the fast group.
††† $P<.001$ with respect to the stimulated samples (PMA).
# $P<.05$ with respect to the corresponding control (DCF).
$P<.001$ with respect to the corresponding control (DCF).

(33 ± 6). At 20 min of incubation, the AI was higher in the slow mice and NAC decreased this capacity in the slow animals without any effect on the fast mice. There were no differences in adherence between groups after 30 min of incubation (data not shown), although NAC reduced adherence in the slow animals (from 71 ± 10 to 56 ± 9 with $P<.01$). Chemotaxis capacity was significantly higher in the fast group with respect to the slow mice. In slow mice, NAC increased this capacity bringing it to the level of the fast group. Ingestion of latex beads was lower in the slow mice as compared to those of the fast group although this difference was not significant. Treatment with NAC increased this function in the slow group.

As regard ROS production, the control groups (slow and fast) did not show any difference in the absence of stimulation. In the presence of PMA, macrophages of fast animals increased their production of ROS but no changes were found in the cells of slow mice, with these macrophages showing lower level of ROS than those of the fast animals. In addition, the treated fast group presented similar levels of ROS to those of the control, while the treated slow mice

Table 5
Effect of in vivo treatment with NAC on macrophage functions from fast and slow Swiss mice

Function	Fast		Slow	
	Control	Treated	Control	Treated
Adherence	44 ± 8	43 ± 9	64 ± 7* * *	47 ± 7†††
Chemotaxis	384 ± 70	408 ± 85	203 ± 32* * *	407 ± 58†††
Phagocytosis	872 ± 169	841 ± 135	775 ± 105	933 ± 98†
ROS production				
DCF	119 ± 23	112 ± 25	92 ± 26	133 ± 30†
DCF + PMA	154 ± 34#	185 ± 23###	118 ± 18 *	181 ± 25#,††

Each value is the mean ± S.D. of eight values corresponding to eight independent experiments. Adherence = expressed as AI of cells incubated 20 min; chemotaxis = expressed as number of macrophage per filter; phagocytosis = expressed as number of latex beads ingested per 100 macrophage; H_2O_2 production = expressed as fluorescence units.
* $P<.05$ with respect to the fast group.
* * * $P<.001$ with respect to the fast group.
† $P<.05$ with respect to the slow and fast controls.
†† $P<.01$ with respect to the slow and fast controls.
††† $P<.001$ with respect to the slow and fast controls.
# $P<.05$ with respect to the nonstimulated samples (DCF).
$P<.001$ with respect to the nonstimulated samples (DCF).

showed an increased ROS production with respect to the slow controls. After incubation with PMA, the macrophages of fast and slow animals increased their ROS production in relation to the corresponding nonstimulated cells. Moreover, macrophages from slow treated animals showed an increase in their stimulated ROS production with respect to those cells of the slow control mice.

3.2.2. Lymphocyte functions

With respect to adherence, no differences were observed between the control groups (slow and fast) for any time of incubation (10, 20, and 30 min), although we could observe a slight increase in this capacity in the slow mice (without statistical significance). NAC decreased the AI of the slow group only at 10 min of incubation and had no effect on the fast animals (Fig. 3).

Regarding chemotaxis (Fig. 3), it was lower in the slow group and increased in the treated animals, especially in the slow mice.

With regard to ROS production, no significant differences between the control groups (fast and slow) were found in the absence of stimulation. Furthermore, the fast control group presented levels of ROS similar to those of the fast treated mice. Conversely, the treated slow mice produced more ROS than the slow controls (73 ± 17 as opposed to 47 ± 10, with $P < .05$). After stimulation with PMA, only the fast treated group showed an increased ROS production with respect to its nonstimulated control (from 58 ± 11 to 92 ± 15 with $P < .01$). In addition, as shown in Fig. 4, the PMA-stimulated lymphocytes of the fast control group presented higher levels of ROS than those of the slow control mice. NAC ingestion increased the stimulated ROS production in both fast and slow groups with respect to untreated (control) animals.

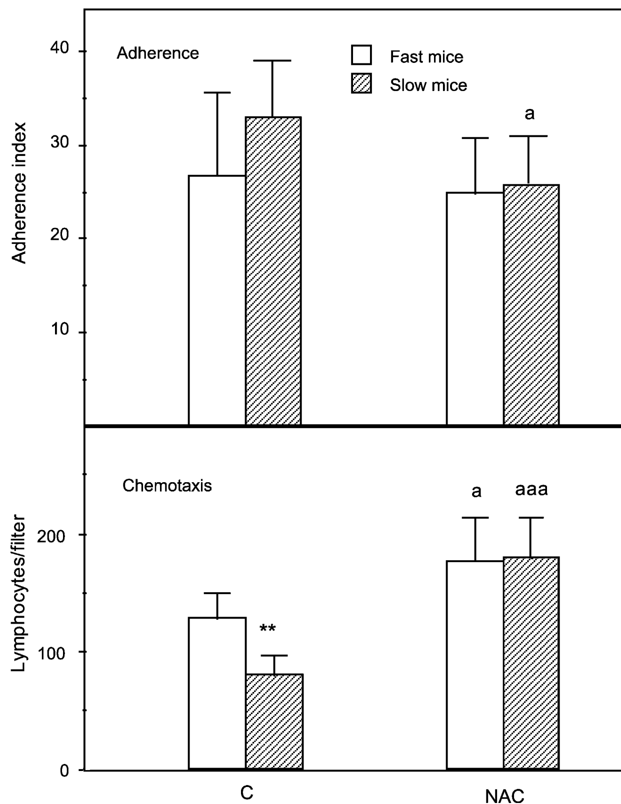

Fig. 3. AI and chemotaxis of peritoneal lymphocytes from control and treated mice. Each column represents the mean ± S.D. of eight values corresponding to eight independent experiments. $^{**}P < .01$ with respect to the fast group; $^{a}P < .05$ and $^{aaa}P < .001$ with respect to slow and fast controls (C).

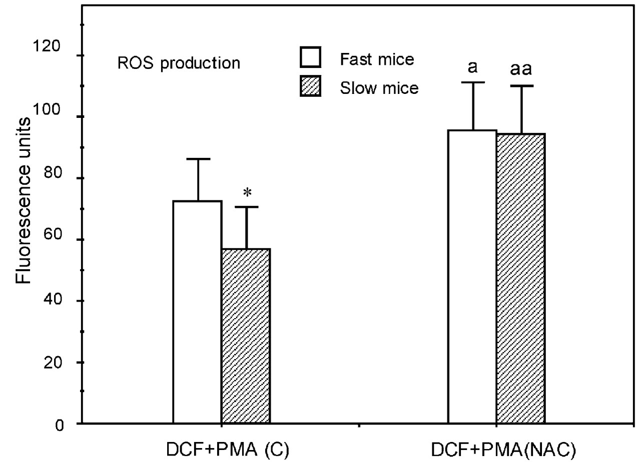

Fig. 4. ROS production by peritoneal lymphocytes from control and treated mice. Each column represents the mean ± S.D. of eight values corresponding to eight independent experiments. $^{*}P < .05$ with respect to the fast group; $^{a}P < .05$ and $^{aa}P < .01$ with respect to slow and fast controls (C).

4. Discussion

The immune and the nervous system and their interaction suffered an age-related impairment (Goya et al., 1999) that is associated with life span (Dellu et al., 1994; Gilad and Gilad, 1995). Accordingly, we have observed in Swiss mice a relation among their behavior in a simple T-maze test, their immune status, and their life span (De la Fuente et al., 1998a,b; Correa et al., 1999a; Guayerbas et al., 2000, in press; Viveros et al., 2001).

The role of ROS in the ageing process is well known, since free radicals and membrane lipid peroxidation increase with age, while the level of antioxidants, especially of glutathione (GSH), decreases, resulting in oxidative stress (Knight, 2000; Meydani et al., 1998). Therefore, exogenous antioxidants may contribute to the fight against age associated deterioration (Heng-long et al., 2000) and they have been proposed as modulators of immune function in the elderly (Knight, 2000; De la Fuente and Víctor, 2000). In agreement with this, the antioxidant NAC, which increases the intracellular levels of GSH (Meyer et al., 1994), improves both macrophage and lymphocyte functions, especially in cells from slow mice, which are biologically older than fast mice.

In relation with macrophage functions, the adherence of these cells was higher and the chemotaxis was lower in the slow than in the fast mice. These facts reveal the oxidative stress suffered by the slow mice, which is related to the increase in adhesion molecules involving activation of the NFκβ transcription factor (Cheng et al., 1998) and the release of MIF (Hirokawa et al., 1998), resulting in a higher adherence and a lower chemotaxis, as previously found in slow mice (Correa et al., 1999a; De la Fuente et al., 1998a,b; Viveros et al., 2001). Moreover, an increase of adherence and a decrease of chemotaxis have been shown in immune cells from old animals or elderly subjects (Ginaldi et al., 1999; McArthur, 1998; Ortega et al., 2000). Antioxidants such as NAC have been reported to inhibit NFκβ transcription factor, increasing chemotaxis, and diminishing adherence (Víctor et al., 1999; Faruqi et al., 1997). In the present work, NAC shows these effects on adherence and chemotaxis in vitro as well as in vivo. Thus, these functions, which are the first steps of the immune response, are improved by this antioxidant, allowing the phagocytes to reach the inflammation sites.

Phagocytic capacity is the next step of the macrophage immune response. We have observed in a previous work a decrease of this function in slow animals (Correa et al., 1999a). It could be expected that the antioxidant stimulates the phagocytic capacity as it happens. In fact, this stimulation is higher in the slow mice, which present lower phagocytic indexes before treatment. We have already observed the beneficial effects of several antioxidants on this function both in vitro (Del Río et al., 1998; Correa et al., 1999b) and in vivo (Correa et al., 1999a; Blanco et al., 1999).

In the presence of a phagocytic stimulus, macrophages initiate what is known as the respiratory burst, characterized by ROS production that shows germicidal capacity (Knight, 2000; Klebanoff, 1980). In a previous work, we have observed increased levels of intracellular superoxide anion in macrophages from adult mice after in vitro treatment with NAC (Del Río et al., 1998). Ingestion of a diet supplemented with thioproline and NAC maintained the ability to produce intracellular ROS in the peritoneal macrophages from adult mice after a phagocytic stimulus (Blanco et al., 1999). On the other hand, extracellular ROS production, which are harmful to cells, was decreased after ingestion of the antioxidant thioproline (Correa et al., 1999a). ROS production of stimulated macrophages from the slow mice is lower in comparison with that from fast animals and is enhanced by NAC in vivo. Thus, this antioxidant could contribute to an appropriate response of macrophages to the phagocytic stimulus. Conversely, NAC in vitro had no effect on ROS production in nonstimulated cells, whereas it decreased the level of these ROS in macrophages stimulated by PMA. These results are in agreement with a direct antioxidant action of NAC in the detoxification of ROS.

Regarding lymphocyte functions, our results are similar to those obtained on macrophages, with lymphocytes from slow mice showing a higher adherence and a lower chemotaxis. These observations are in agreement with previous studies of our group on aged mice (De la Fuente et al., 1998a,b, 1993). The effects of NAC were similar to those observed on macrophages, with a decrease in adherence and an increase in chemotaxis, probably through the same mechanisms. ROS act in these cells as intracellular second messengers, involved with the activation of lymphocytes (Suzuki and Ono, 1999). ROS production was lower in PMA-stimulated lymphocytes from slow mice, and NAC ingestion increased this PMA-induced production, possibly providing the cell with the appropriate oxidant/antioxidant balance. NAC in vitro acted directly, decreasing the levels of ROS previously stimulated by PMA.

In summary, our results support that NAC acts directly on the leukocyte functions studied and that its ingestion could be favorable to the immune function. This improvement may be more relevant in situations of oxidative stress such as the ageing process. This seems to be the case for the slow mice, which show an early immunosenescence that could be prevented to certain degree by NAC.

Acknowledgements

We thank Dr. Jaime Miquel by his critical reading of the manuscript. This work was supported by FISss (97/2078), CM (08.5/0061/2001.1), and MCyT (BFI 2001-1218) grants.

References

Blanco B, Ferrández MD, Correa R, Del Río M, Guaza C, Hernanz A, De la Fuente M. Changes in several functions of murine peritoneal macrophages by *N*-acetylcysteine and thioproline ingestion. Comparative effect between two strains of mice. BioFactors 1999;10:179–85.

Boyden SV. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. J Exp Med 1962;115:453–6.

Burgess W, Liu Q, Zhou JH, Tang Q, Ozawa A, Vanhoy R, Arkins S, Dantzer R, Kelley KW. The immune–endocrine loop during aging: role of growth hormone and insulin-like growth factor I. Neuroimmunomodulation 1999;6(1–2):56–68.

Chatterjee A, Deb S. Genotoxic effect of arecoline given either by the peritoneal or oral route in murine bone marrow cells and the influence of *N*-acetylcysteine. Cancer Lett 1999;139(1):23–31.

Cheng JJ, Wung BS, Chao YJ, Wang DL. Cyclic strain-induced reactive oxygen species involved in ICAM-1 gene induction in endothelial cells. Hypertension 1998;31:125–30.

Correa R, Blanco B, Del Río M, Víctor VM, Guayerbas N, Medina S, De la Fuente M. Effect of a diet supplemented with thioproline on murine macrophage function in a model of premature aging. BioFactors 1999a; 10:195–200.

Correa R, Del Río M, De la Fuente M. Improvement of murine immune function in vitro by thioproline. Immunopharmacology 1999b;44: 281–91.

De la Flora S, Cesarone CF, Balansky R. Chemoprotective properties and mechanism of *N*-acetylcysteine. The experimental background. J Cell Biol 1995;22:33–41.

De la Fuente M, Víctor VM. Anti-oxidants as modulators of immune function. Immunol Cell Biol 2000;78:49–54.

De la Fuente M, Del Río M, Ferrández MD, Hernanz A. Modulation of phagocytic function in murine peritoneal macrophages by bombesin, gastrin-releasing peptide and neuromedin C. Immunology 1991;73:205–11.

De la Fuente M, Ferrández MD, Muñoz F, De Juan E, Miquel J. Stimulation by the antioxidant thioproline of the lymphocyte function of old mice. Mech Ageing Dev 1993;68:27–36.

De la Fuente M, Ferrández MD, Del Río M, Burgos MS, Miquel J. Enhancement of leukocyte functions in aged mice supplemented with the antioxidant thioproline. Mech Ageing Dev 1998a;104:213–25.

De la Fuente M, Miñano M, Víctor VM, Del Río M, Ferrández MD, Díez A, Miquel J. Relation between exploratory activity and immune function in aged mice: a preliminary study. Mech Ageing Dev 1998b;102:263–77.

De la Fuente M, Medina S, Del Río M, Ferrández MD, Hernanz A. Effect of ageing on the modulation of macrophage functions by neuropeptides. Life Sci 2000;67:2125–35.

Dellu F, Mayo W, Vallee M, Le Moal M, Simon H. Reactivity to novelty during youth as a predictive factor of cognitive impairment in the elderly: a longitudinal study in rats. Brain Res 1994;653:51–6.

Del Río M, Ruedas G, Medina S, Víctor VM, De la Fuente M. Improvement by several antioxidants of macrophage function in vitro. Life Sci 1998;63(10):871–81.

Fabris N. Neuroendocrine–immune interactions: a theoretical approach to aging. Arch Gerontol Geriatr 1991;12:219–30.

Faruqi RM, Poptic EJ, Faruqi TR, De la Motte C, DiCorleto PE. Distinct mechanism for *N*-acetylcysteine inhibition of cytokine-induced E-selectin and VCAM-1 expression. Am Physiol Soc 1997;273:H817–26.

Ferrández MD, Correa R, Del Río M, De la Fuente M. Effects in vitro of several antioxidants on the natural killer function of aging mice. Exp Gerontol 1999;34:675–85.

Gilad GM, Gilad VH. Strain, stress, neurodegeneration and longevity. Mech Ageing Dev 1995;78:75–83.

Ginaldi L, De Martinis M, D'Ostilio A, Marini L, Loreto MF, Quaglino D. Immunological changes in the elderly. Aging: Clin Exp Res 1999;11(5):281–6.

Gosset P, Wallaert B, Tonnel AB, Fourneau C. Thiol regulation of the production of TNF-alpha, IL-6 and IL-8 by human alveolar macrophages. Eur Respir J 1999;14(1):98–105.

Goya RG, Brown OA, Bolognani F. The thymus–pituitary axis and its changes during aging. Neuroimmunomodulation 1999;6(1–2):137–42.

Guayerbas N, Sánchez AI, Gamallo A, Miquel J, De la Fuente M. Mouse performance in an exploratory activity test as a longevity biomarker. Animal research and welfare. A partnership. London: Laboratory Animals Ltd., 2000. p. 159–61.

Guayerbas N, Puerto M, Víctor VM, Miquel J, De la Fuente M. Leukocyte function and life span in a murine model of premature immunosenescence. Exp Gerontol 2002;37:249–56.

Guayerbas N, Catalán M, Víctor VM, Miquel J, De la Fuente M. Relation of behaviour and macrophage function to life span in a murine model of premature immunosenescence. Behav Brain Res [in press].

Heng-long H, Forsey RJ, Blades TJ, Barrat MEJ, Parmar P, Powell JR. Antioxidants may contribute in the fight against ageing: an in vitro model. Mech Ageing Dev 2000;121:217–30.

Heussler VT, Fernandez PC, Machado J, Botteron C, Dobbelaere DAE. *N*-Acetylcysteine blocks apoptosis induced by *N*-alpha-tosyl-L-phenylalanine chloromethyl ketone in transformed T-cells. Cell Death Differ 1999;6(4):342–50.

Hirokawa K. Age-related changes of signal transduction in T cells. Exp Gerontol 1999;34(10):7–18.

Hirokawa K, Sakaue S, Furuya Y, Ishii J, Hasekawa A, Tagami S, Kawakami Y, Sakai M, Nishi S, Nishihira J. Tumor necrosis factor-alpha regulates the gene expression of macrophage migration inhibitory factor through tyrosine kinase-dependent pathway in 3T3-L1 adipocytes. J Biochem 1998;123:733–9.

Klebanoff SJ. Oxygen intermediates and the microbicidal events. Mononuclear phagocytes. Functional aspects. The Hague: Martinus Nijhoff, 1980.

Knight JA. Review: free radicals, antioxidants and the immune system. Ann Clin Lab Sci 2000;30(2):145–58.

McArthur WP. Effect of aging on immunocompetent and inflammatory cells. Periodontology 2000 1998;16:53–79.

Meydani SN, Santos MS, Wu D, Hayek MG. Antioxidant modulation of cytokines and their biological function in the aged. Z-Ernahrungswiss 1998;37:35–42.

Meyer A, Buhl R, Magnussen H. The effect of oral *N*-acetylcysteine on lung glutathione levels in idiopathic pulmonary fibrosis. Eur Respir J 1994;7:431–6.

Ortega E, Barriga C, De la Fuente M. Aging and non-specific immune response. Facts and research in gerontology. Paris: Albarède-Vellas, Serdi, 1993.

Ortega E, García JJ, De la Fuente M. Aging modulates some aspects of the non-specific immune response of murine macrophages and lymphocytes. Exp Physiol 2000;85(5):519–25.

Pahlavani MA, Richardson A. The effect of age on the expression of interleukin-2. Mech Ageing Dev 1996;89:125–54.

Pawelec G. Immunosenescence: impact in the young as well as the old? Mech Ageing Dev 1999;108:1–7.

Sahnoun Z, Jamoussi K, Zeghal KM. Free radicals: fundamental notions and methods of exploration. Thérapie (Paris) 1998;53(4):315–39.

Solana R, Pawelec G. Molecular and cellular basis of immunosenescence. Mech Ageing Dev 1998;102:115–29.

Suzuki Y, Ono Y. Involvement of reactive oxygen species produced via NADPH oxidase in tyrosine phosphorylation in human B and T lineage lymphoid cells. Biochem Biophys Res Commun 1999;255:262–7.

Víctor VM, De la Fuente M. *N*-Acetylcysteine improves in vitro the function of macrophages from mice with endotoxin-induced oxidative stress. Free Radic Res 2002;36(1):33–45.

Víctor VM, Guayerbas N, Garrote D, Del Río M, De la Fuente M. Modulation of murine macrophage function by *N*-acetylcysteine in a model of endotoxic shock. BioFactors 1999;10:347–57.

Viveros MP, Fernández B, Guayerbas N, De la Fuente M. Behavioral characterization of a mouse model of premature immunosenescence. J Neuroimmunol 2001;114:80–8.

Wayne SJ, Rhyne RL, Garry PJ, Goodwin JS. Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. J Gerontol 1990;45:M45–8.