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Improvement of Leukocyte Functions in Young Prematurely Aging Mice After a 5-Week Ingestion of a Diet Supplemented with Biscuits Enriched in Antioxidants

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ABSTRACT

In our previous studies, diet supplementation with therapeutic thiolic antioxidants improved the function of peritoneal leukocytes from mice, especially in adult prematurely aging mice (PAM). In the present work, we have studied the effects of ingestion during 5 weeks of a diet supplemented with 20% (wt/wt) of biscuits enriched with antioxidants (vitamin C, vitamin E, β-carotene, zinc, and selenium) on several immune functions of peritoneal leukocytes from young PAM. The results show that, in macrophages, chemotaxis and phagocytosis as well as the intracellular free radical levels, which are depressed in PAM in comparison with the control non-prematurely aging mice (NPAM), increase after supplementation, especially in the PAM. An increase also occurs in lymphocyte chemotaxis, proliferative response to the mitogen concanavalin A, and interleukin-2 release, as well as in natural killer cell activity. However, the release of tumor necrosis factor-α, which increases with aging, decreases after 5 weeks of supplementation. As a well preserved function of the immune system is an excellent marker of health and longevity, the improvement of leukocyte functions after ingestion of the present diet suggests that this antioxidant supplementation may be useful for the preservation of health and functional longevity in aging populations. *Antioxid. Redox Signal.* 7, 1203–1210.

INTRODUCTION

AGING is associated with a decline of many physiological functions, including those of the immune system (30, 36), and its impairment exerts a great influence on age-related morbidity and mortality. In fact, leukocyte functions have been proposed as biomarkers of longevity (44). Accordingly, a fact that confirms the key role of immune function in health and longevity is that the centenarians who reach that very advanced age in good health are those showing a perfect preservation of leukocyte functions, with values like those of adult subjects (25). Thus, the concept of "biological age," which arises as a consequence of the different rates of physiological changes in the members of a population of the same chronological age (4), is useful to assess the level of senescence of each individual, and therefore his life expectancy. To calculate the biological age, several parameters should be determined, and in this respect our group, using a model of premature aging in the mouse, has shown a relation between the immune competence of a subject and its life span. This model relies on the differences in performance among adult mice of the same sex and chronological age when subjected to a behavioral (exploration) test in a simple T-maze. We have observed that the animals that fail the test are "biologically older," *i.e.,* suffer premature immunosenescence (17, 43) and show a neurochemistry similar to that of an older chronological age (21) and higher levels of anxiety and emotionality (43) than the animals of the same chronological age that performed the test correctly [non–prematurely aging mice (NPAM)]. The prematurely

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aging mice (PAM) showed values characteristic of animals of an older chronological age for several immune function parameters investigated, particularly those of the phagocytic function in macrophages. The confirmation that these parameters are markers of biological age was provided by the fact that the PAM had a significantly shorter life span than the NPAM (27, 28). Until now, PAM had been detected in adult mouse populations, but the model had not been characterized in young mice.

As the levels of endogenous antioxidants decrease in oxidative stress situations such as aging, diet supplementation with antioxidants may be useful to prevent pathological aging and to increase longevity (13). Further, the oxidative and inflammatory processes are closely related, and the immune cells are an important source of both oxidant and proinflammatory compounds needed to support their functions (1, 13). These compounds can become a source of tissue damage if their levels are not controlled by the antioxidant defenses of the cell or antioxidant supplements (7, 31). Previous studies on adult and old experimental animals, as well as on adult and elderly men and women, have demonstrated the beneficial effects on the immune functions, *in vitro* and *in vivo*, of several types of antioxidants (2, 3, 7–11, 13, 15, 18, 19, 23, 24, 34, 39, 42, 45). Moreover, the antioxidants increase the functions that are impaired and decrease those that are very stimulated, thus acting as modulators of the immune function (14). Recently, we have observed that diet supplementation with thiolic antioxidants, such as *N*-acetylcysteine (NAC) and thioproline (TP), improves the immune function in PAM, bringing it to NPAM values (29, 38), particularly as regards macrophage functions and, according to our unpublished data, increases mouse life span.

Despite the above favorable effects of antioxidants on the immune function, diet supplementation with antioxidant compounds in order to improve health has become controversial due to several studies, mainly performed in humans, that show no beneficial effect as regards decrease in infectious morbidity after several types and doses of antioxidant intake (6, 32). Recently, it has been suggested that more research is needed on this subject (7, 34) and that the use of multinutrient supplements containing optimum amounts of essential trace elements and vitamins with antioxidant properties may be the most effective way to enhance the immune system functions and prevent infections (7). As oxidative stress has been detected in young subjects (37), the aim of the present work was to test the effect of the ingestion for 5 weeks of a diet supplemented with biscuits enriched with antioxidants on several functions of peritoneal leukocytes from young PAM and NPAM.

MATERIALS AND METHODS

Animals

We have used young female ICR (CD-1) mice (*Mus musculus*) (Harlan Ibérica, Barcelona, Spain), which were 8 weeks old on arrival at our laboratory. The mice were specific pathogen-free, as tested by Harlan according to FELASA recommendations. The animals were randomly divided into groups of five, and each group was housed in a polyurethane box, at a constant temperature (22 ± 2°C) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France), on a 12-h/12-h reversed light/dark cycle. All animals were fed water and standard Sander Mus (A.04 diet from Panlab L.S., Barcelona, Spain) pellets *ad libitum*. The diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals. Mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC.

Experimental groups

The animals were marked for their individual follow-up. At 9 weeks of age, the spontaneous exploratory behavior of each mouse was tested in a T-shaped maze, as previously described (27). The test is performed by placing the mouse 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 PAM (which required >10 s, each of the four times, to complete the exploration of the first arm) from the NPAM (which completed that exploration in 10 s or less). The animals showing an intermediate response to the T-maze were removed from the study. Thus, we had two groups of animals: one group contained the NPAM population and the other the PAM population, with a NPAM/PAM ratio of 100:0 and 0:100, respectively. This test was always performed between 9:00 and 11:00 a.m., under red light.

The NPAM and PAM were randomly divided into control and treated groups. The control group received standard diet for maintenance (A.04 diet from Panlab L.S., Barcelona, Spain), and the treated group received 80% control diet plus 20% biscuits enriched with nutritional doses of antioxidants like vitamin C, vitamin E, β-carotene, zinc, and selenium (Table 1). The treatment was maintained for 5 weeks and then, at the age of 18 ± 2 weeks, samples were obtained to evaluate the immune function of peritoneal leukocytes.

TABLE 1. NUTRITIONAL COMPOSITION OF THE DIETS USED

Diets	*Energy (kcal)*	*Carbohydrates (g/100 g)*	*Fat (g/100 g)*	*Proteins (g/100 g)*	*Vitamin C (mg/100 g)*	*Vitamin E (mg/100 g)*	*β-Carotene (mg/100 g)*	*Zinc (mg/100 g)*	*Selenium (μg/100 g)*
Control (A.04)	290	60.0	3.1	16.1	0	3.0	0	2.4	5.0
Biscuits	458	62.3	5.7	14.5	69.3	19.2	3.4	7.7	73.0

Collection of peritoneal leukocytes

The peritoneal suspensions were obtained between 8:00 and 10:00 a.m., without sacrificing the animals and following the method previously described (27). Macrophages, identified by morphology and nonspecific esterase staining, were counted and then adjusted by dilution with Hanks' solution to 5×10^5 macrophages/ml in the samples in which we assayed the macrophage functions. The cellular viability, determined in each experiment using the trypan-blue exclusion test, was in all cases >95%.

Assays of phagocytic function in peritoneal macrophages

In the peritoneal suspensions, with macrophages adjusted to 5×10^5 cells/ml of Hanks' solution, we carried out the study of some of the different steps of the phagocytic process, *i.e.,* mobility to the infectious focus (chemotaxis), phagocytosis of foreign material, and digestion capacity of this material through the production of intracellular free radicals, namely, superoxide anion, which is the first response in the respiratory burst, and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2).

The chemotaxis assays were performed according to a modification (16) of the original technique described by Boyden (5), which consists basically of the use of chambers with two compartments separated by a filter (Millipore, Bedford, MA, U.S.A.). The peritoneal suspensions were deposited in the upper compartment of the Boyden chambers, and f-met-leu-phe (Sigma, St. Louis, MO, U.S.A.) (a positive chemotactic peptide *in vitro*) was placed in the lower compartment. After a 3-h incubation, the filters were fixed and stained, and the chemotaxis index (C.I.) was determined by counting the total number of macrophages in one third of the lower face of the filters.

The latex phagocytosis assay was carried out following the method described by De la Fuente (12). The peritoneal suspensions were incubated in culture plates (Sterilin, Teddington, U.K.) for 30 min with latex beads [1.09 μm diluted to 1% phosphate-buffered saline (PBS); Sigma]. The particles ingested by 100 macrophages were counted and expressed as phagocytic index. Moreover, the number of ingesting macrophages per 100 macrophages was also determined and expressed as phagocytic efficiency.

Superoxide anion production was evaluated by assessing the capacity of this anion, produced by macrophages, to reduce nitroblue tetrazolium (NBT; Sigma). This was carried out following the method described by De la Fuente (12) and slightly modified (27). The peritoneal suspensions were mixed with NBT (1 mg/ml in PBS) and latex beads (stimulated samples) or Hanks' solution (nonstimulated samples). After a 60-min incubation, the samples were centrifuged. The intracellular reduced NBT was extracted with dioxan (Sigma) and, after centrifugation, the supernatant absorbance at 525 nm was determined (intracellular superoxide anion levels). The results are expressed as nanomoles per 10^6 cells using a pattern curve.

The ROS production was measured by fluorimetry following a method previously described (38). 2′,7′-dichlorofluorescin diacetate (DCF-DA) was used as a probe because it is oxidized in the cytoplasm by ROS to 2′,7′-dichlorofluorescein, which is a highly fluorescent compound. The peritoneal suspension (adjusted to 10^6 leukocytes/ml with Hanks' solution) was incubated with DCF-DA (1 m*M*) and phorbol myristate acetate (PMA; 50 ng/ml) (stimulated samples) or Hanks' solution (nonstimulated samples). Finally, the samples were analyzed by fluorimetry. The results are expressed as the stimulation index, which is the percentage of stimulation in response to PMA, with the value of the nonstimulated samples being 100%.

Assay of tumor necrosis factor-α (TNF-α) release from peritoneal leukocytes in culture

TNF-α was measured in the supernatants of cultures of peritoneal leukocytes. Cell suspensions were incubated following the method previously described (22) using lipopolysaccharide (LPS; *E. coli,* 055:B5; Sigma; 1 μg/ml in well), and after 48 h of incubation, the levels of TNF-α were measured using an enzyme-linked immunosorbent assay (ELISA) kit (HyCult Biotechnology, b.v., Uden, The Netherlands). The results are expressed as picograms per milliliter.

Assays of peritoneal lymphocyte function

Proliferation assay. A previously described method was used (22). The peritoneal suspension was adjusted at a final concentration of 5×10^5 cells/ml in complete medium (RPMI-1640, PAA, Pasching, Austria; plus 10% fetal calf serum, Life Technologies; plus 1% gentamicin, PAA), and aliquots of 200 μl were dispensed in plates of 96 wells (Orange Scientific, Braine-l'Alleud, Belgium). Then concanavalin A (Con A; Sigma; 1 μg/ml in well) (stimulated samples) or complete medium (nonstimulated samples) was added, and plates were incubated for 48 h. Subsequently, 5 μCi of [^{3}H]-thymidine was added to each well; after 8 h, the cells were harvested in a semiautomatic microharvester (Skatron, Norway) and thymidine uptake was measured in a β counter (Perkin-Elmer, MA, USA) for 1 min. The results were expressed as the stimulation index, which is the percentage of stimulation in response to Con A, with the values for the nonstimulated samples being 100%.

Lymphocyte chemotaxis assay. The lymphocyte chemotaxis capacity was evaluated as described above for macrophages.

Interleukin-2 (IL-2) release assay. The levels of IL-2 were determined in culture supernatants of the above-described peritoneal leukocyte cultures. After a 48-h incubation with the mitogen Con A, the supernatants were collected and measured using an ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.). The results are expressed as picograms per milliliter.

Cytotoxicity assay. An enzymatic colorimetric assay was used for cytolysis measurements of target cells (Cytotox 96 TM Promega, Madison, WI, USA) based on the determination of lactate dehydrogenase using tetrazolium salts, as previously used by us on this type of samples (24). Murine lymphoma YAC-1

cells were used as target in the natural killer (NK) assay. The cells were maintained in complete medium. Target cells were seeded in 96-well U-bottom culture plates (Orange Scientific) at 10^4 cells/well in 1640 RPMI without phenol red (PAA). Effector cells, *i.e.,* peritoneal leukocytes, were added at 10^5 cells/well, with the effector/target rate 10:1. The plates were incubated for 4 h, and lactate dehydrogenase enzymatic activity was measured in 50 µl/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 of target cells, the following equation was used: % lysis = $(E - ES - TS) / (M - ES - TS) \times 100$, where E is the mean of absorbances in the presence of effector cells, ES is the mean of absorbances of effector cells incubated alone, TS is the mean of absorbances in target cells incubated with medium alone, and M is the mean of maximum absorbances after target cells were incubated with lysis solution. The results are expressed as % lysis.

Statistical analysis

The data are expressed as the mean ± SD of n values corresponding to the same number of experiments. Each value is the mean of the data from an assay performed in duplicate. The data were examined statistically by one-way analysis of variance to evaluate the treatment in both the NPAM and the PAM groups and to investigate the differences between control NPAM and PAM. The minimum significance level was $p < 0.05$. The normality of the samples was confirmed by the Kolmogorov–Smirnov test and the homogeneity of variances by the Levene test.

RESULTS

Macrophage functions

Figure 1 shows the results of some of the most important macrophage functions studied. The chemotaxis capacity, represented by the C.I. (Fig. 1A), was decreased in the PAM in comparison with the NPAM group ($p < 0.001$) and was increased by the treatment in the PAM group ($p < 0.001$), whereas no effect was observed in the NPAM group. The phagocytic efficiency (Fig. 1B) presented similar levels in both the NPAM and the PAM groups. However, after the treatment there was a significant increase ($p < 0.05$) of this function in both groups. The statistical analysis of the phagocytic index (Fig. 1C) rendered significant differences ($p < 0.05$) between the PAM and the NPAM group, with the PAM showing the lower values. Moreover, this function increased strikingly ($p < 0.001$) with the treatment in the PAM, whereas similar values were found in the control and the treated NPAM group.

Figure 2 shows the intracellular free radical superoxide anion values (under latex beads-stimulated conditions), which is the first response in the respiratory burst (Fig. 2A), and PMA-stimulated ROS (H_2O_2 chiefly) (Fig. 2B). [The nonstimulated intracellular superoxide anion levels were 35 ± 8 (control NPAM), 38 ± 7 (treated NPAM), 21 ± 6 (control PAM), and 38 ± 8 (treated PAM)]. The control PAM group showed a significant decrease of this free radical with respect to the control NPAM group ($p < 0.05$). The treated NPAM presented levels

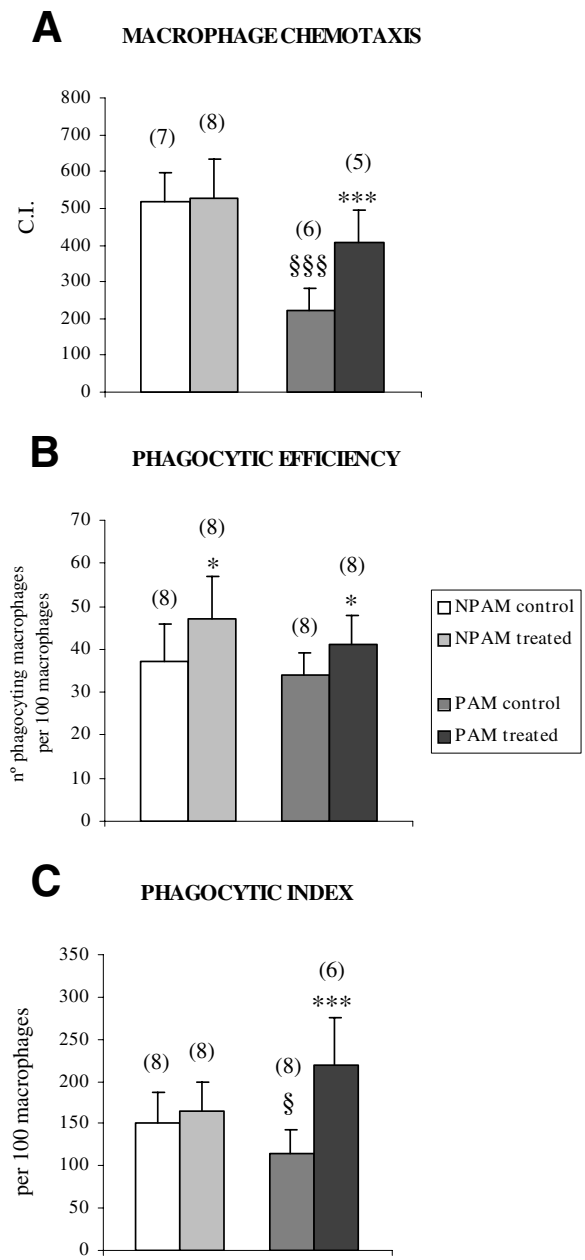

FIG. 1. Macrophage chemotaxis (C.I. = number of macrophages/filter) (A), phagocytic efficiency (B), and phagocytic index (C) of peritoneal macrophages from female ICR (CD-1) control and treated NPAM and PAM. Bars represent the mean ± SD of n values (in brackets), corresponding to the same number of experiments, after 5 weeks of treatment. Each value is the mean of data from an assay performed in duplicate. §$p < 0.05$, §§§$p < 0.001$ compared with the control NPAM. *$p < 0.05$; ***$p < 0.001$ compared with the respective control NPAM or PAM.

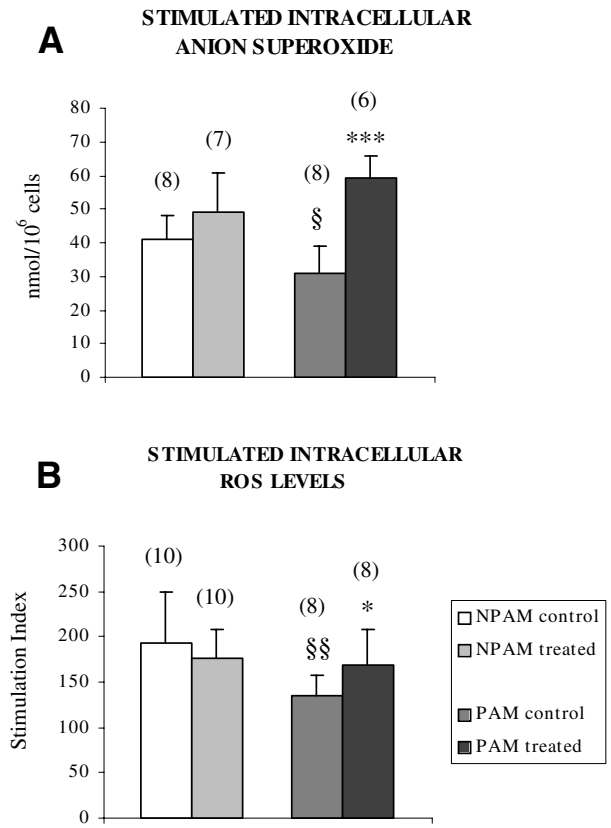

FIG. 2. Stimulated intracellular superoxide anion (nmol/10^6 cells) (A) and PMA-stimulated intracellular ROS levels (stimulation index) (B) of peritoneal macrophages from female ICR (CD-1) control and treated NPAM and PAM. [Stimulation index: expressed as the percentage of stimulation with respect to the nonstimulated values, which are 100%. The nonstimulated fluorescence units are: 5,253 ± 1,332 (control NPAM), 7,719 ± 1,802 (treated NPAM), 9,615 ± 2,328 (control PAM), and 4,690 ± 1,355 (treated PAM)]. Bars represent the mean ± SD of *n* values (in brackets) corresponding to the same number of experiments, after 5 weeks of treatment. Each value is the mean of data from an assay performed in duplicate. §$p < 0.05$; §§$p < 0.01$ compared with the control NPAM. *$p < 0.05$; ***$p < 0.001$ compared with the respective control NPAM or PAM.

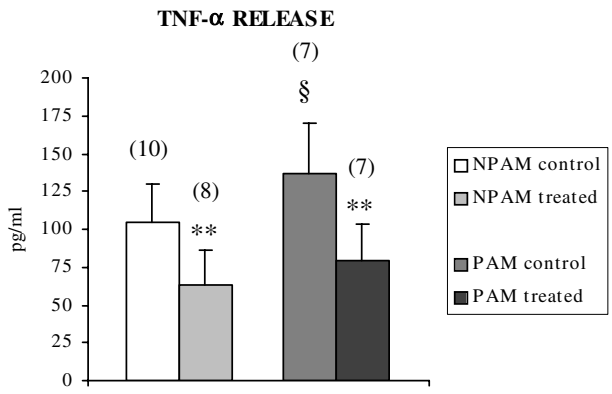

FIG. 3. Release of TNF-α (pg/ml) from LPS-stimulated (1 μg/ml) cultures of peritoneal leukocytes from female ICR (CD-1) control and treated NPAM and PAM. Bars represent the mean ± SD of *n* values (in brackets) corresponding to the same number of experiments, after 5 weeks of treatment. Each value is the mean of data from an assay performed in duplicate. §$p < 0.05$ compared with the control NPAM. **$p < 0.01$ compared with the respective control NPAM or PAM.

similar to those found in the control NPAM group, whereas the treated PAM values increased strikingly with respect to the control PAM group ($p < 0.001$). ROS were decreased in the control PAM group in comparison with the control NPAM group ($p < 0.01$). The statistical analysis on the effect of the treatment in both groups revealed an increase of this function in the treated PAM ($p < 0.05$), with no change in the NPAM group.

Moreover, we also measured one of the most important inflammatory mediators released by the macrophages, namely, TNF-α (pg/ml) (Fig. 3), from LPS-stimulated (1 μg/ml) peritoneal leukocyte cultures. The stimulation indexes of LPS-stimulated lymphoproliferation were 306 ± 33 (control NPAM), 295 ± 23 (treated NPAM), 203 ± 40 (control PAM), and 201 ± 42 (treated PAM). No differences were observed between groups. In regard to TNF-α release, the control PAM group showed higher levels ($p < 0.05$) of this inflammatory mediator than those of the control NPAM group. Furthermore, the treatment was effective in both PAM and NPAM, with the treated groups showing lower values ($p < 0.01$).

Lymphocyte functions

Figure 4 shows the results of the peritoneal lymphocyte functions studied. The Con A-stimulated lymphoproliferation, represented by the stimulation index (Fig. 4A), suffered a decrease in the control PAM with respect to the control NPAM group ($p < 0.01$). The statistical analysis demonstrated that the treatment increased strikingly the values found in the PAM group in comparison with those of the control PAM group ($p < 0.001$), whereas no difference was found between the control and the treated NPAM group. The chemotaxis capacity, measured as the C.I. (number of lymphocytes/filter) (Fig. 4B), was lower in the control PAM group than in the control NPAM group ($p < 0.001$), and was significantly stimulated ($p < 0.05$) by the treatment in PAM. No difference between the control and the treated NPAM group was observed. As regards IL-2 release (Fig. 4C), the control PAM showed a striking decrease with respect to the control NPAM ($p < 0.01$) and a strong increase after the treatment ($p < 0.001$). However, no difference was found between control and treated NPAM. Finally, the natural killer (NK) activity (Fig. 4D) showed lower levels in the control PAM group than in the control NPAM group ($p < 0.001$) and a striking increase after the treatment ($p < 0.001$) in PAM, showing levels similar to those found in the control NPAM group. The treatment was also effective in the treated NPAM group, which showed a significant increase ($p < 0.01$) in comparison with the control NPAM.

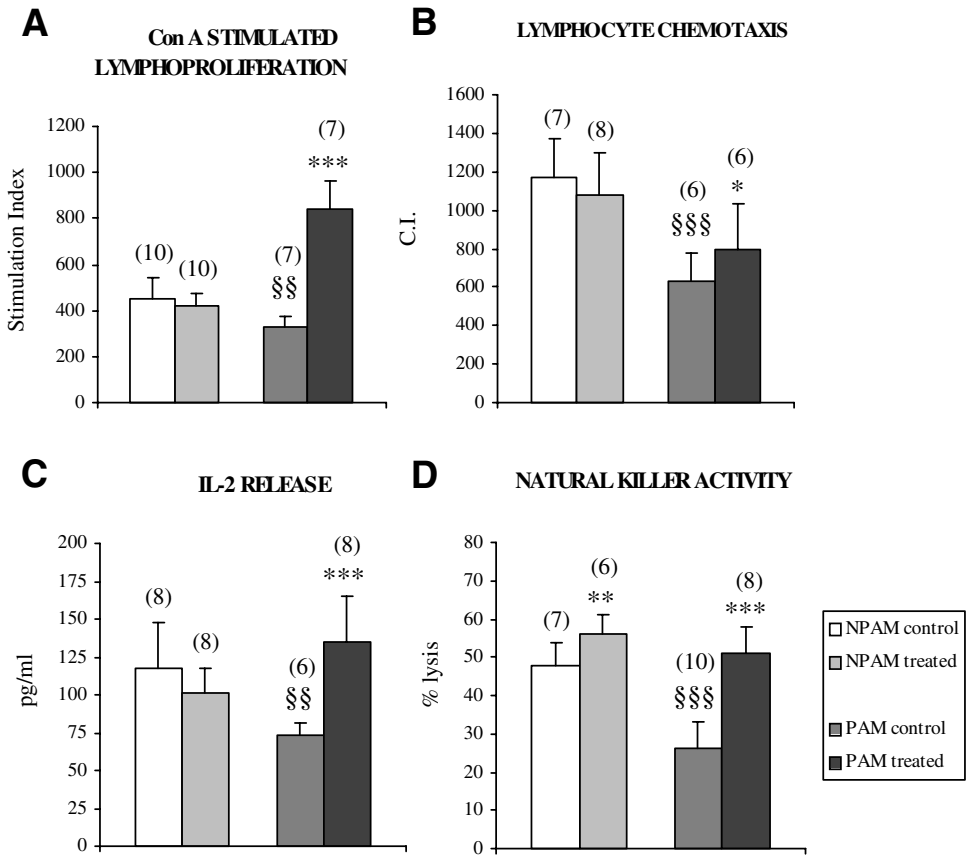

FIG. 4. Con A-stimulated lymphoproliferation (stimulation index) (A), lymphocyte chemotaxis (C.I. = number of lymphocytes/filter) (B), IL-2 release (pg/ml) from Con A-stimulated (1 μg/ml) cultures of peritoneal leukocytes (C), and NK activity (% lysis) (D) of peritoneal lymphocytes from female ICR (CD-1) control and treated NPAM and PAM. [Stimulation index: expressed as the percentage of the values after stimulation with respect to the nonstimulated values, which are 100%. The nonstimulated cpm are: 1,175 ± 287 (control NPAM), 2,159 ± 442 (treated NPAM), 1,329 ± 335 (control PAM), and 1,057 ± 231 (treated PAM)]. Bars represent the mean ± SD of *n* values (in brackets) corresponding to the same number of experiments, after 5 weeks of treatment. Each value is the mean of data from an assay performed in duplicate. §§$p < 0.01$, §§§$p < 0.001$ compared with the control NPAM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the respective control NPAM or PAM.

DISCUSSION

The present work is the first in which we have observed that it is possible to extend the model of premature aging to young animals, thus detecting PAM and NPAM at an early chronological age. Moreover, the functions investigated in peritoneal leukocytes show statistically significant differences between young PAM and NPAM, with the PAM showing a worse immune function than the NPAM. Thus, the PAM seem to have a chemotaxis capacity of macrophages and lymphocytes, phagocytic index and digestion (measured by the intracellular levels of superoxide anion and ROS) of the macrophages, lymphoproliferative response to Con A (a typical T-cell mitogen), IL-2 release, and NK activity lower than those of the NPAM. All these findings are characteristic for older subjects (27, 30, 35, 36). Moreover, TNF-α, a proinflammatory cytokine produced mainly by macrophages, increases with age (13, 28) and is released at higher levels in the PAM than in the NPAM. Thus, it seems that young PAM are immunologically older than NPAM. As we have shown in previous work a worse macrophage function in adult PAM than in NPAM of the same age [which was accompanied by a shorter life span in the PAM (27)], it is probable that the young PAM have a shorter life expectancy than the NPAM.

The free radical theory of aging is based on the adverse effects of oxidative stress, which is known to increase with age (33, 40). However, according to recent work high levels of oxidative stress have also been observed in younger subjects (37). The oxidant–antioxidant balance is critical for immune cell function (31) and, accordingly, a rapidly increasing amount of data shows that the use of antioxidants *in vitro* and *in vivo* has favorable effects on the immune cell function of young (10, 11, 15, 19, 39, 42) and specially of aged subjects (6–9, 13, 18, 24, 34, 45). We have previously shown an improvement of the immune functions of adult PAM after the ingestion for 5 weeks of a diet supplemented with thiolic antioxidants such as NAC and TP (9, 29, 38). In the present study, the ingestion of a diet supplemented with moderate amounts of several antioxidants (vitamin C, vitamin E, β-carotene, zinc, and selenium) improves all the immune functions studied in PAM. In NPAM, the effect of the supplementation is only observed in the phagocytic efficiency, the

TNF-α release, and the NK activity. It is evident that if the young NPAM have an adequate immune function, no effect or only a discrete effect may be shown after antioxidant supplementation. Moreover, we have observed that the amount of thiolic antioxidant ingestion needed to improve several immune functions is higher in aged than in adult mice (20).

In general, the functions studied in the PAM after supplementation become similar or better than those in the NPAM. This is a usual result obtained with modulators of immune function such as antioxidants (14), *i.e.,* that the effects are more evident in subjects showing an initial worse function (14, 18). These results are quite interesting because it is essential that macrophages show an adequate capacity in their defensive role against infecting microorganisms (7), and because the phagocytic functions mentioned above have been suggested as biomarkers of longevity in the PAM (27). The supplementation tested in the present work improves macrophage function and therefore could enhance the immune response and reduce the incidence of infections, with resulting improvement of health and longevity in the young PAM. In a recent review, Chandra (7) suggested that the use of multinutrient supplements containing optimum antioxidant levels is very appropriate to reduce infections and improve immune responses. All antioxidants of the biscuits used in the present study have been shown to enhance several immune functions and are the most widely used as dietary antioxidants. Thus, ascorbic acid improves immune functions such as those studied here (7, 13, 15, 34, 42), and the same applies to vitamin E (7, 13, 19, 34). As *in vitro,* these antioxidants produce similar effects on the functions investigated, such as chemotaxis (23), it is probable that the favorable effects of diet supplementation can be directly linked to the greater amount of these antioxidants in the enriched diet. Indeed, β-carotene and selenium play an important role in improving the immune function (8, 32, 45). Zinc has also been proposed as a very important contributor to effective immune system functions, but too much or too little zinc can induce immunologic dysfunctions (3, 7, 11, 39). In general, the amounts of antioxidants are as important as the biological age of the subject who ingests them, which should be taken into consideration in order to explain some controversial results obtained with antioxidant supplementation (7, 34, 41). Although several mechanisms involved in the action of the antioxidants on the immune cells may be different for the various antioxidants, the effects of all the above antioxidants on the functions of these cells are very similar (26). As it is possible to show an older biological age coexisting with a very young chronological age in the same mouse, and as an antioxidant diet supplementation such as indicated above is not hazardous, our data suggest that diet enrichment with the moderate amounts of natural antioxidants used in the present study may be useful even when administered at a young age. As an optimal antioxidant protection is probably associated with a longer life span, diet supplementation with foods rich in antioxidants starting at a young age could decrease the rate of aging and help to reach a healthy longevity.

ACKNOWLEDGMENT

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ABBREVIATIONS

C.I., chemotaxis index; Con A, concanavalin A; DCF-DA, 2′,7′-dichlorofluorescin diacetate; ELISA, enzyme-linked immunosorbent assay; H_2O_2, hydrogen peroxide; IL-2, interleukin-2; LPS, lipopolysaccharide; NAC, *N*-acetylcysteine; NBT, nitroblue tetrazolium; NK, natural killer; NPAM, non–prematurely aging mice; PAM, prematurely aging mice; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TP, thioproline.

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