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Neutrophils of Centenarians Show Function Levels Similar to Those of Young Adults

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OBJECTIVES: To analyze several functions and antioxidant parameters of peripheral blood neutrophils from healthy centenarians (men and women) and compare them with those of healthy young (aged 25–35) and middle-aged (aged 65–75) men and women.

DESIGN: Cross-sectional study.

SETTING: Community-based.

PARTICIPANTS: Twenty-one healthy centenarians (8 men), 30 young adults (15 men), and 30 middle-aged adults (15 men).

MEASUREMENTS: Several neutrophil functions (adherence, chemotaxis, phagocytosis, and stimulated and nonstimulated intracellular superoxide anion levels) and antioxidant parameters (glutathione levels and catalase activity) were measured in peripheral blood neutrophil suspension in the three study groups.

RESULTS: Neutrophil functions of the middle-aged group were worse than those of young adults and centenarians (lower chemotaxis and phagocytosis and higher adherence and superoxide anion levels). The neutrophil functions of the centenarians were closer to those of the young adults. Age-related differences in neutrophil functions were fundamentally similar in men and women, except for intracellular superoxide anion levels, which were lower in young adult women than in young adult men. With normal aging, total glutathione levels decrease, but the centenarians in this study showed levels similar to those of young adults. Centenarians showed the highest catalase activity of the three groups.

CONCLUSION: Progressive impairment of the immune system accompanies aging. The better preservation of function and antioxidant systems in the neutrophils of centenarians could play a key role in the longevity of these subjects. **J Am Geriatr Soc 56:2244–2251, 2008.**

Key words: centenarians; neutrophils; immunosenescence; antioxidants; oxidative stress

Immunosenescence, the age-related impairment of immune function, increases susceptibility to infection, cancer, and autoimmune diseases in old mammals, including humans,[1] with the result that morbidity and mortality also rise. It has been proposed that the functional capacity of the immune cells is a marker of health and longevity.[2,3] It has been shown that several immune functions are markers of biological age and longevity, because animals with premature immunosenescence of those functions show a significantly shorter life span.[4–8] By contrast, animals that reach very old age have immune parameters similar to those of younger adult animals.[9]

Continuous adaptation of the body, including the immune system, to impairments that occur over time accompanies aging, with some immune mechanisms deteriorating and others being maintained. These adaptive changes are apparent in healthy human centenarians, who represent the best living example of successful aging,[10] with well-preserved leukocyte functions.[11]

Age-related changes in lymphocytes have been well studied, whereas findings on the effect of aging on phagocytic cell function and redox state are not consistent in the scientific literature,[11] and there are few data on centenarians.[12] Moreover, women have a longer life span than men, which has a biological basis,[13] and immune functions show differences according to sex,[14] although these differences have not been studied in centenarians.

It has been proposed that the high rate of infection in aged individuals could be the result of neutrophil dysfunction.[15] Phagocytic cells, especially neutrophils, act in the early phase of defense against infecting microorganisms. Neutrophils are the first cells to participate in the phagocytic process, which involves adherence to the vascular endothelium, migration to the focus of infection (chemotaxis), and phagocytosis of invading microorganisms, which is the

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most representative and relevant function of these cells. Several studies point to a decline in the phagocytic capacity of aged subjects,[4–6] especially in neutrophils.[16–19]

In the phagocytic process, the destruction of ingested material frequently involves the production of oxygen-free radicals, the first of which is superoxide anion, a precursor of active microbicidal oxidants. All cells, including immune cells, require adequate levels of antioxidant defenses to prevent damage to biomolecules caused by the excess of free radicals. Moreover, leukocytes are particularly sensitive to oxidative damage because of the high content of polyunsaturated fatty acids in their membranes and the genetic expression required for their function.[20] Thus, in neutrophils, maintaining appropriate levels of antioxidants is important to avoid oxidation, because successful aging may depend on better ability to cope with oxidative stress.

It was found that, to a large extent, age-related changes in immune function are due to the oxidative stress to which immune function is exposed over time.[7] Thus, in peritoneal leukocytes from mice, production and release of oxidant compounds increase with age, whereas the level of antioxidant defenses decreases. As a consequence of this oxidative stress, an increase in lipid peroxidation and oxidative damage of deoxyribonucleic appears in those cells affected by aging.[21,22] Extreme longevity, as observed in centenarians, is associated with a lower degree of oxidative stress than that experienced by middle-aged adults.[23,24]

It is generally difficult to detect solely age-dependent changes in the immune system; consequently, the literature sometimes presents conflicting data. Factors such as chronic disease, nutrition, and lifestyle have a profound effect on immunity and thus conceal more-subtle age-related changes.[25] To overcome this problem, one study,[26] working under the SENIEUR EURAGE protocol, set strict inclusion criteria for human immunogerontology studies that include clinical information, laboratory data, and immunopharmacological interferences.

In view of the above, the aim of the present study was to analyze the most-representative functions of peripheral blood neutrophils in healthy centenarians and to compare them with those of healthy young and middle-aged adults. Moreover, because there is a difference in the average life span according to sex, these functions were also compared between men and women in the three age groups. In addition, two antioxidant parameters that are closely related to longevity—glutathione levels and catalase activity—were studied in centenarians and middle-aged and young adult subjects. The results could provide further evidence of the usefulness of these determinations as markers of "biological age" and, therefore, predictors of survival in humans.

METHODS

Subjects

Eighty-one subjects were studied (Table 1). Twenty-one were centenarians. Physical and mental health status were evaluated as recommended by the SENIEUR protocol[26] for immunogerontological studies. The two younger control groups were volunteers selected according to the same criteria of the SENIEUR protocol. The first group contained 30 young adult subjects who were laboratory personnel and students. The second control group contained 30 middle-aged subjects selected from retired personnel of *Hospital Clínico San Carlos*. This study was performed with the informed consent of the donors and was approved by the *Hospital Clínico San Carlos* ethics committee.

Table 1. Clinical and Functional Characteristics of the Subjects Studied

Characteristic	Young Adults (n = 30)	Middle-Aged Adults (n = 30)	Centenarians (n = 21)
Age, mean (range)	28 (25–35)	69 (65–75)	101 (100–102)
Male, n (%)	15 (50.0)	15 (50.0)	8 (38.1)
Living at home, %	100	100	90.5
Barthel Index score 80–100, %	100	100	71
Mini-Mental State Examination score > 25, %	100	90	78
Number of chronic conditions, mean (range)	0 (0–0)	1.7 (0–5)	3.6 (0–6)
Number of drugs, mean (range)	0 (0–0)	2.1 (0–7)	2.3 (0–5)

Peripheral blood samples (10 mL) were collected using venipuncture and put into sodium citrate-buffered Vacutainer tubes (BD Diagnostics – Preanalitical Systems, Madrid, Spain) (always in the morning to avoid circadian changes in immune function) and were analyzed during the following 24 hours.

Identification and Quantification of Immune Cells

The polymorphonuclear neutrophils (PMNs) were isolated from whole blood using the Histopaque 1.077/1.119 (Sigma, St. Louis, MO) double-density gradient centrifugation method at 700 *g* for 30 minutes. The PMNs (in the lower halo) were washed in phosphate buffered saline (PBS) (Sigma) solution and then counted and adjusted by diluting them in Hank's buffered salt solution to 1×10^5 cells/mL. The viability of the PMNs was 95% (as determined using trypan blue staining). Cells were incubated at 37°C in a sterile humidified atmosphere of 5% carbon dioxide (CO_2).

Adherence Capacity

The adherence capacity was measured following a method described previously,[27] with later modifications.[28] Briefly, 1 mL of whole blood (diluted 1:1 with Hank's medium) was placed in a Pasteur pipette into which 50 mg of nylon fiber was packed to a length of 1.25 cm. After 10 minutes, the effluent had drained by gravity. The adherence index (AI) percentage was calculated as follows:

$$AI = [(\text{PMNs/mL diluted} - \text{PMNs/mL of effluent samples})/(PMNs/mL)\,diluted)] \times 100$$

This measure of adherence using nylon fibers is similar to that used for vascular endothelium.[27]

Chemotactic Capacity

Chemotactic capacity was evaluated using a modification[29] of an original technique described previously,[30] which measures the mobility of neutrophils toward an infectious focus. Aliquots of 0.3 mL of the neutrophil suspension (10^5 neutrophils/mL) were deposited in the upper compartment of a Boyden chamber separated by a nitrocellulose filter (Millipore, Milford, MA) with a pore diameter of 3 μm, and fMet-Phe-Leu (Sigma) was added to the lower compartment at 10^{-8} M as a chemoattractant agent. After 3 hours of incubation at 37°C in 5% CO_2, the filter was fixed (methanol 50%) and stained (Diff-Quick pack; Dade, Düdingen, Switzerland). The chemotactic index, which represented the total number of neutrophils counted using optical microscopy (immersion objective) on 16 random optical microscope fields, was calculated.

Phagocytosis Assay

Phagocytosis assay was performed following a method described previously,[31] with later modifications[29] for ingestion of inert particles (latex beads). Aliquots of 200 μL of neutrophil suspension were incubated on migration inhibition–factor plates (Sterilin, Teddington, UK) for 30 minutes. The plates were washed with PBS at 37°C to obtain an adherent monolayer, and 20 μL of latex beads (1.09 μm diluted to 1% PBS) were added. After 30 minutes of incubation, the plates were washed, fixed (methanol 50%), and stained with the Diff-Quick pack. The phagocytic index (PI) (number of latex beads ingested per 100 neutrophils) and the phagocytic efficiency (PE) (percentage of neutrophils with ≥1 phagocytosed particles) was determined using optical microscopy.

Measurement of Superoxide Anion Levels

The superoxide anion level—the first response in the respiratory burst that starts the destruction of ingested microorganisms—was evaluated by assessing its capacity to reduce nitroblue tetrazolium (NBT).[32] The assay was performed following a method described previously,[29] modified as follows. Aliquots of 250 μL of neutrophil suspension were mixed with 250 μL of NBT (1 mg/mL in PBS) and a suspension of 50 μL of latex beads (1.09 μm diluted to 1% PBS) (stimulated samples) and 50 μL of Hank's solution (nonstimulated samples). After 60 minutes of incubation at 37°C, the reaction was stopped, and the samples were centrifuged, the supernatants were discarded, and the reduced NBT was extracted using dioxane (Sigma). Supernatant absorbance at 525 nm was determined in a spectrophotometer using dioxane as a blank control. The results were expressed as mmol/10^6 neutrophils by extrapolating from a standard curve of NBT reduced with 1,4-dithioerythritol (Boehringer Mannheim, Mannheim, Germany).

Antioxidant Parameters: Glutathione Levels and Catalase Activity

Antioxidant parameters (glutathione levels and catalase activity) were determined only in individuals with enough remaining neutrophils after the neutrophil function assays.

Total contents of glutathione were determined using the Tietze enzymatic assay, with some modifications.[21] The reaction was monitored spectrophotometrically at 412 nm for total glutathione determination. The results were expressed as nmols per 10^6 cells.

Catalase enzymatic activity was measured following a method described previously,[33] with some modifications, and based on lower absorbance at 240 nm because of the decomposition of hydrogen peroxide (H_2O_2) by the enzyme. Aliquots of 1 mL of the neutrophil suspension, adjusted to 10^6 cells/mL, were used to perform the enzymatic assay. The cells were centrifuged at 1,076 *g* for 10 minutes at 4°C, and the pellets were resuspended in 50 mM of phosphate buffer containing 14 mM of H_2O_2 (Merck, Darmstadt, Germany). The samples were then sonicated and centrifuged at 3,200 *g* for 20 minutes at 4°C. The enzymatic assay was followed spectrophotometrically at 240 nm through the decomposition of H_2O_2 into H_2O+O_2. The results were expressed as U/10^6 cells.

Statistical Analysis

SPSS 10.0 (SPSS, Inc., Chicago, IL) was used for the statistical analysis of the results. All data are expressed as medians and interquartile ranges except sex, which is expressed as absolute numbers, and age, which is expressed as the mean with a range. The number of experiments is presented within parentheses for each experimental group and parameter measured in the different figures. Each value is the mean of data from an assay performed in duplicate. The values from the three groups were compared using the Kruskal-Wallis and Mann-Whitney tests for independent variables. $P < .05$ was considered significant.

RESULTS

Results are expressed as medians and interquartile ranges (IQRs) in the three age groups (centenarian (n = 21, 8 men), middle-aged adult (n = 30, 15 men), and young adult (n = 30, 15 men)). In the figures, the data are divided according to sex. A subgroup (n = 6, 2 men and 4 women) was found in the middle-aged subjects in which the values were more similar to those of the young adult group than to those of the middle-aged group. The medians and interquartile ranges for this subgroup are indicated.

Adherence and Chemotaxis

Figure 1 shows the results for adherence capacity (expressed as the AI (Figure 1A)) and of chemotaxis (expressed as the chemotaxis index (Figure 1B)) of neutrophils in the three age groups studied.

In normal aging, adherence is greater with older age ($P < .01$), although the centenarians showed significantly lower adherence ($P < .01$) than the middle-aged subjects and no difference from the adult subjects. No differences were found according to sex for each age group. In all three groups, men showed greater adherence with age (the value in young adults (median 40, IQR 33–47) was lower than in middle-aged subjects (median 53, IQR 40–60) $P < .05$), although the centenarians had significantly lower adherence (median 23, IQR 17–46; $P < .01$) than middle-aged subjects and no difference from young adult subjects. In women, there were no differences in the three groups studied (adults: median 42, IQR 26–60; middle-aged: median 58, IQR 45–69; and centenarians: median 53, IQR 35–55). In the

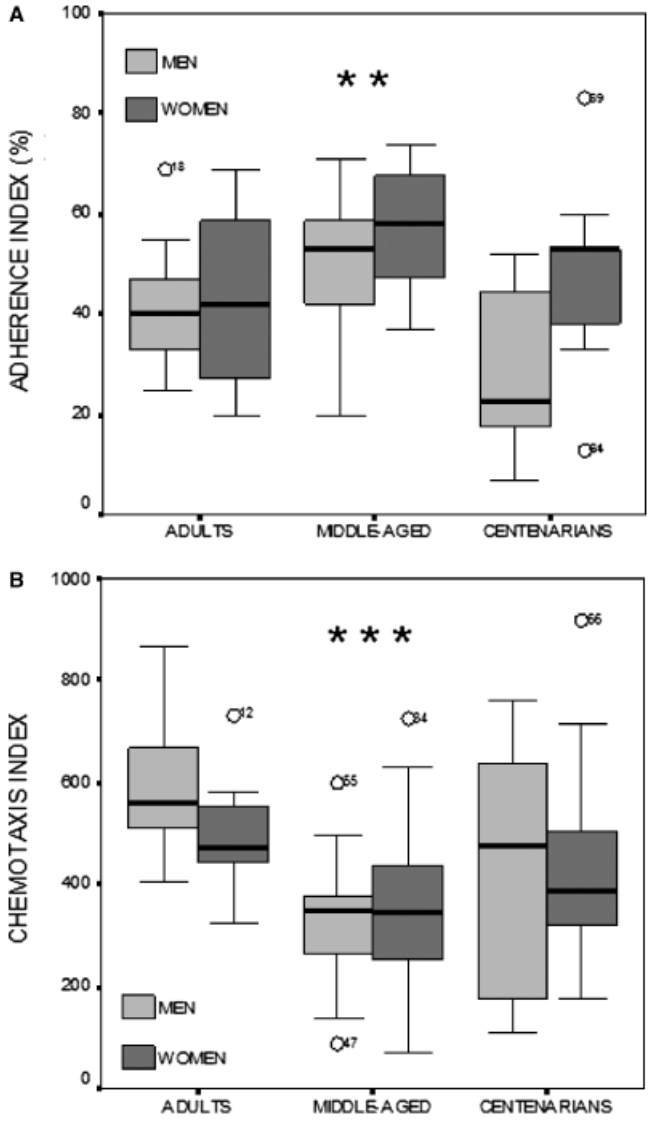

Figure 1. (A) Adherence index in the three study groups. Adherence in centenarians (n = 21; median 44, interquartile range (IQR) 19–53) was lower than in middle-aged subjects (n = 30; median 54, IQR 44–67) ($P < .01$). There were no differences between centenarians and young adults (n = 30; median 41, IQR 28–51) ($P = .73$). (B) Chemotaxis index in the three study groups. Chemotaxis in middle-aged subjects (n = 30; median 348, IQR 245–415) was lower than in centenarians (n = 21; median 456, IQR 259–566 ($P = .37$)) and young adults (n = 30; median 538, IQR 467–621) ($P < .001$). There were no differences between centenarians and young adults ($P = .21$). $^{**}P < .01$, $^{***}P < .001$ in comparison with young adults.

middle-aged subgroup, the median and IQR were 39 and 29 to 43.

Chemotaxis was lower in older subjects ($P < .001$ young adults vs middle-aged subjects), and centenarians showed statistically nonsignificantly higher values than the middle-aged group. There was no influence of sex in any of the three age groups (middle-aged men (median 350, IQR 240–392) and women (median 347, IQR 246–491) had lower values than young adults (men, mean 558, IQR 498–702; women, median 474, IQR 424–559)) and no differences from centenarians (men, median 478, IQR 145–680; women, median 388, IQR 297–538). Values in the middle-aged subgroup were median 552 and IQR 496 to 653.

Phagocytosis

The phagocytic capacity of neutrophils (Figure 2), measured as the PI (Figure 2A) and PE (Figure 2B), decreases with aging. Thus, the values in cells from the middle-aged group were significantly lower ($P < .001$) than those in the young adult group. The centenarians showed higher values

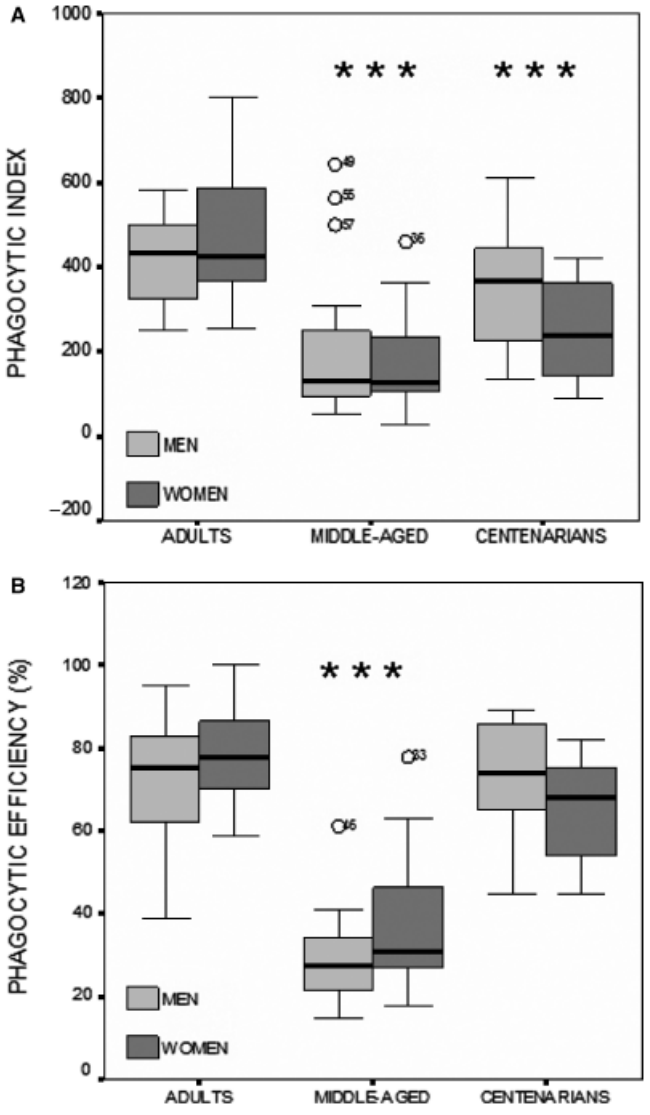

Figure 2. (A) Phagocytic index in the three study groups. The phagocytic index in the middle-aged group (n = 30; median 129, interquartile range (IQR) 96–305) was lower than in the young adult group (n = 30; median 427, IQR 324–562) ($P < .001$) and in centenarians (n = 21; median 249, IQR 163–403) ($P < .01$). There were differences between these groups ($P < .001$). (B) Phagocytic efficiency in the three study groups. Phagocytic efficiency in the middle-aged group (n = 30; median 30, IQR 23–40) was lower than in the young adult group (n = 30; median 76, IQR 63–87) ($P < .001$). The centenarians (n = 21; median) had a significantly higher phagocytic index than the middle-aged group 70, IQR 55–80 ($P < .001$). There were no differences between centenarians and young adults ($P = .09$). $^{**}P < .01$, $^{***}P < .001$ in comparison with young adults.

than the middle-aged subjects (PI, $P<.01$; PE, $P<.001$), with no difference from the young adult group in PE.

The influence of sex was studied in each age group, but no differences were found. In addition, PI and PE were lower ($P<.01$ and $P<.001$, respectively) in middle-aged (median 130, IQR 82–310 and median 28, IQR 22–35, respectively) than in young adult (median 431, IQR 324–536 and median 75, IQR 61–87, respectively) and centenarian (median 368, IQR 224–446 and median 74, IQR 63–86, respectively) men, with no differences between centenarian and young adult men.

When women were analyzed, lower levels ($P<.001$) were found in both parameters (PI and PE) in middle-aged (median 127, IQR 100–303 and median 31, IQR 26–51, respectively) than in young (median 424, IQR 350–594 and median 78, IQR 69–87, respectively) adults. Centenarian women had higher PE levels (median 68, IQR 52–77) than middle-aged women ($P<.001$), and no differences were found in PI (median 237, IQR 142–383) from middle-aged women. Centenarian women had lower levels on both parameters than young adults (PI, $P<.001$ and PE, $P<.05$).

In the middle-aged subgroup, the values were PI, median 480, IQR 354 to 583 and PE, median 57, IQR 49 to 67.

Levels of Superoxide Anion

Nonstimulated and stimulated levels of superoxide anion (Figure 3A and B, respectively) seemed to increase with aging ($P<.01$). Centenarians had lower levels than middle-aged subjects ($P<.01$ in the nonstimulated samples, $P<.05$ in the stimulated samples), with no differences from young adults. When the influence of sex on each age group was analyzed, no differences were found for the nonstimulated or stimulated samples, except in the adult group ($P<.01$). The influence of sex in the three age groups was investigated. There were higher superoxide anion levels in nonstimulated and stimulated samples in middle-aged women (median 104, IQR 44–132 and median 128, IQR 61–168, respectively) than in young adult (median 41, IQR 30–52 and median 58, IQR 41–75, respectively) and centenarian (median 50, IQR 27–62 and median 67, IQR 54–92, respectively) women, with no differences between centenarian and young adult women. No differences were apparent between the male groups (nonstimulated and stimulated samples: median 61, IQR 49–87 and median 82, IQR 69–95 in adult men; median 66, IQR 48–129 and median 84, IQR 70–164 in middle-aged men; and median 51, IQR 30–68 and median 68, IQR 52–121 in centenarian men). In the middle-aged subgroup, the values were median 29, IQR 23 to 31 in the nonstimulated samples and median 55, IQR 49 to 63 in the stimulated samples.

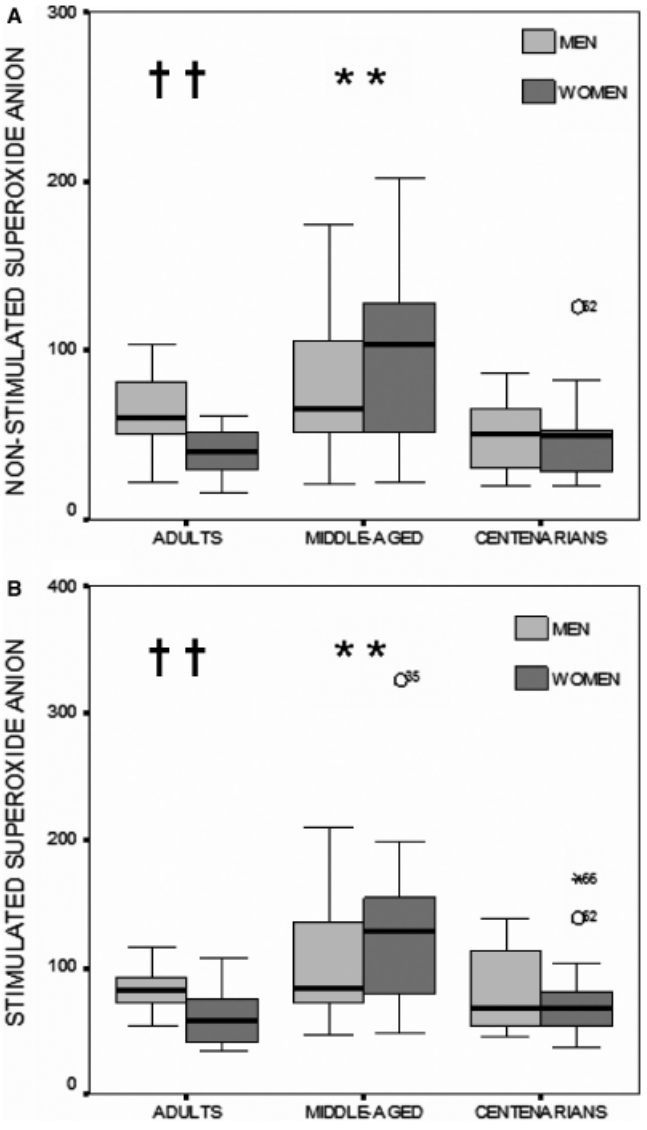

Figure 3. Intracellular levels of superoxide anion (O_2^-) in nonstimulated samples (A) and samples stimulated with latex beads (B) (nmol/10^6 cells). Intracellular O_2^- levels in middle-aged subjects (n = 30) in both samples (nonstimulated: median 74, interquartile range (IQR) 47–130 and stimulated: median 104, IQR 68–165) were higher than in young adults (median 52, IQR 34–64 and median 75, IQR 52–88) ($P<.01$) and centenarians (median 50, IQR 29–66, $P<.01$ and median 67, IQR 54–99, $P<.05$). *$P<.05$; **$P<.01$; ***$P<.001$ in comparison with young adults. $^{\dagger\dagger}P<.01$ between men and women in control young adults.

Antioxidant Parameters

Total glutathione levels (Figure 4A) decreased with aging ($P<.001$), although centenarians (n = 12) had a significantly higher value ($P<.001$) than middle-aged subjects (n = 12) and no differences from young adult subjects (n = 12).

Catalase activity (Figure 4B) showed no differences with aging (n = 6 in both groups), although greater activity in this antioxidant enzyme was observed in the neutrophils from centenarians (n = 8) than in those from the control groups ($P<.001$).

DISCUSSION

To the authors' knowledge, this work is the first to study the typical functions of peripheral blood neutrophils of healthy centenarians and to compare them with those of healthy younger subjects. The results of the present study show that centenarians (men and women) have better neutrophil function than middle-aged men and women, with levels closer to those of young adult subjects. The antioxidant

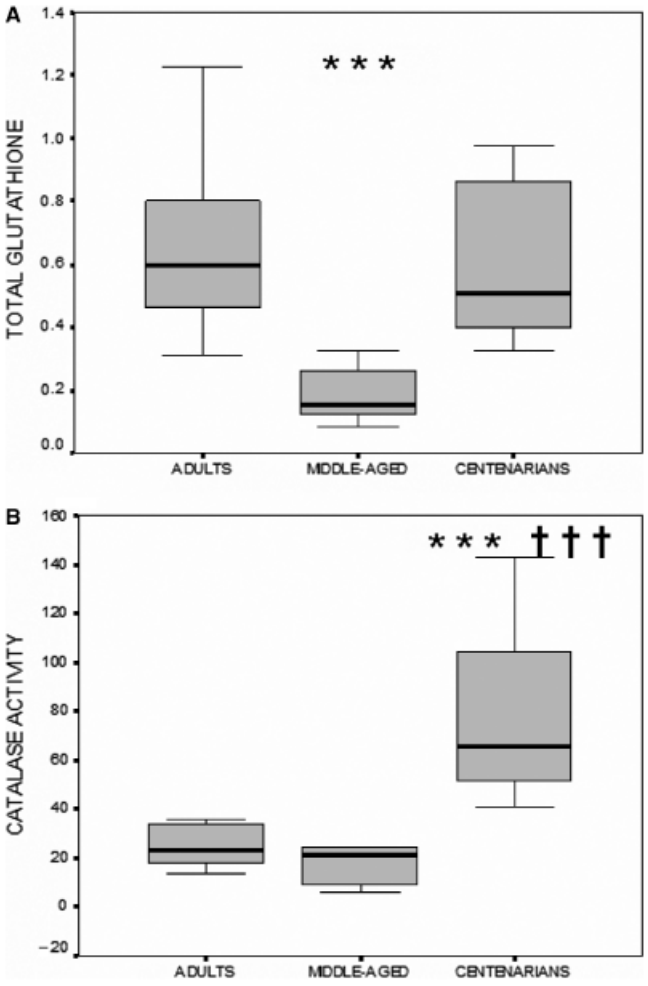

Figure 4. Levels of antioxidants in the three study groups. (A) Total glutathione levels. Middle-aged subjects had lower glutathione levels (n = 12; median 0.16, interquartile range (IQR) 0.12–0.27) than young adults (n = 12; median 0.60, IQR 0.46–0.81) and centenarians (n = 12; median 0.51, IQR 0.40–0.88) ($P < .001$). (B) Catalase activity. Centenarians had the highest catalase activity (n = 8; median 66, IQR 49–114) of the three groups. There was no difference between young (n = 6; median 24, IQR 17–36) and middle-aged (n = 6; median 21, IQR 8–25) adults ($P = .39$). ***$P < .001$ in comparison with young adults. $^{\dagger\dagger\dagger}P < .001$ in comparison with middle-aged adults.

parameters studied, such as glutathione and catalase activity, are also better preserved in neutrophils from centenarians than in those from middle-aged subjects.

To focus specifically on the target subjects and render the results easier to understand, a healthy population was selected.[26] This also allowed confounders, such as chronic disease and medication, which could alter immune status and, consequently, mask the aging effect, to be avoided.

The results obtained in the present study show an important age-associated alteration of neutrophil functions that was not present in the centenarian group. Thus, neutrophils from middle-aged subjects showed greater adherence to endothelium and lower chemotaxis capacity (Figure 1), that is, an impaired neutrophil function that was not present in the centenarians, because their neutrophil activity was similar to that of adults. Greater adherence and lower chemotaxis, as was observed in phagocytic cells, such as macrophages, from mice accompany aging.[6,21,34] Previous studies have also shown greater adhesion of neutrophils from elderly subjects[7,19] and lower chemotactic activity of these cells.[7,16,17,19,35] When neutrophils are very high adherent to endothelium and have very low migration capacity toward the infectious focus, they cannot perform their function appropriately. The increase in adhesion molecule expression[36] and plasma membrane fluidity associated with aging and the altered functions observed[37] were related to greater oxidative stress.[7,38] In addition, an association between low chemotaxis and high lipid peroxide levels with high mortality has been observed.[16] These data suggest that increased adherence and reduced chemotaxis of neutrophils with age could contribute to the higher risk of infection in elderly people.[39] Moreover, peritoneal macrophages from prematurely aging mice (PAM) show greater adherence and lower chemotaxis than nonprematurely aging mice (NPAM) of the same chronological age, and PAM have a shorter life span than NPAM.[8] The present work is the first to study these functions of neutrophils in centenarians, and the results show maintenance of adherence and chemotaxis of neutrophils in these subjects at levels similar to those of adults.

With respect to the ingestion capacity of phagocytic cells, an age-related decrease in this function has been observed in macrophages from experimental animals[6,21] and in neutrophils from humans.[7,19,35] Lower ingestion capacity in phagocytic cells would allow greater development of infection, and previous results have shown that a reduced life span accompanies impaired phagocytosis by peritoneal macrophages from PAM.[4,6,8] According to the present work, phagocytic capacity decreases with age, but this did not occur in the centenarian group, whose levels were closer to those of the young adult group (Figure 2). The neutrophils of centenarians maintained all the above functions to an extent similar to young adult subjects and better than the middle-aged group. This better immune condition could contribute to extreme longevity.

Superoxide anion production by neutrophils is necessary for the digestion of ingested material, but excessive production of this free radical is often harmful when it is not compensated by antioxidant defenses, because it may induce not only bacterial killing, but also tissue damage if it is present at high levels.[40] Several studies have found higher levels of superoxide anions in macrophages from old mice than from young adult mice,[4] as well as in neutrophils from elderly subjects.[19] In the present study, superoxide anion levels increased with aging, and centenarians had similar levels to those of young adults. Moreover, lower levels of reactive oxygen species have been reported in longer-living strains of houseflies and mice.[41,42] It was also observed that young adult women had significantly lower levels of superoxide anions than young adult men. This difference is lost when they become elderly, showing a trend toward higher levels in middle-aged women. Higher levels of superoxide anions were observed (unpublished data) in neutrophils from middle-aged women (aged $\geq$50) than in young adult women (aged 30). Recent studies report that the greater longevity of mammalian females, including humans, is associated with better antioxidant capacity.[13,43] This antioxidant capacity, observed in the mitochondria, could be associated with circulating estrogen levels, because estrogen is able to improve antioxidant capacity.[44]

Although no significant differences have been found between centenarian men and women, men had better values than women in all the neutrophil functions studied. Sex differences in the health status of centenarians have been reported, indicating that centenarian men are less heterogeneous and healthier than centenarian women. Because mean longevity in men is lower than in women, men who reach very old age represent a survival collective in better condition than women of the same age. Immunological factors regarding the age-related increase in proinflammatory status and the frequency of human leukocyte antigen ancestral haplotypes also show sex differences that probably contribute to the difference in longevity between men and women.[45]

As for antioxidant parameters, total glutathione levels in neutrophils decrease with aging.[46] In the current study, peripheral blood neutrophils from centenarians showed levels of this important antioxidant that were similar to those of adult subjects. High total glutathione concentrations in blood have been related to excellent physical and mental health in longevous women[47] and low total glutathione levels in blood have been reported in elderly subjects and patients with chronic diseases.[46] With respect to catalase activity, neutrophils from centenarians show the highest antioxidant enzyme activity. Some studies have found a relationship between catalase activity and higher longevity,[48] which could be related to the important role this enzyme plays in tolerance to oxidative stress and adaptive cell response (because it acts only in the presence of high levels of H_2O_2). There are reports of a plasmatic antioxidant status maintained in people aged 90 and older at a level similar to that of the control population (23–66 years);[49] therefore, the antioxidant status shown by centenarians could be an important advantage in avoiding altered age-associated redox status.

A chronic low-grade inflammatory state clearly shown by the increase in serum levels of inflammatory mediators accompanies aging. A wide range of factors have been claimed to contribute to this state, although chronic antigenic stress, which affects the immune system throughout life, with a progressive activation of phagocytic cells, seems to play the most important role.[7,50] This proinflammatory status, known as "inflamm-aging,"[50] which interacts with the genetic background, has the potential to trigger the onset of age-related inflammatory diseases such as atherosclerosis[45] and other conditions. The current study found well-preserved antioxidant status in peripheral blood neutrophils, which act in the earlier phases of infectious diseases and are responsible for initiating the inflammatory cascade. When inflammatory processes escape these antioxidant regulatory mechanisms, systemic effects may be provoked (such as increasing inflammatory cytokines and other oxidative stress molecules), and these could contribute to many chronic age-associated diseases and to higher mortality. The centenarians studied had well-preserved antioxidant ability, low superoxide anion production, and nonaltered peripheral blood neutrophil functions. Therefore, these characteristics could be goals that would enable them to avoid age-associated diseases and attain longevity. The oxidative stress of the immune cells seems to be the basis of their functional deterioration with aging.[7,8] Therefore, it is possible that the redox state of neutrophils provides centenarians with better health and prevents age-related diseases, in agreement with the oxidant–inflammatory theory of aging.[7]

A small subgroup of the middle-aged subjects showed values in all neutrophil functions that were similar to those of young adults. A prospective study of these individuals will tell us whether this subgroup achieves very old age.

CONCLUSION

Because immune function is a marker of health,[2] and animals with premature immunosenescence show a shorter life span,[8] whereas mice reaching very old age have immune functions more similar to those of adult animals,[9] the functional parameters studied in neutrophils may be useful markers of "biological age" and predictors of survival. Moreover, when the functions of phagocytic cells are better in old mice, the longevity of the animals increases.[8] This opens the door to new ways of improving immune function and maybe of increasing mean life span. Further studies are needed to support these ideas and understand better the immune basis of the privileged aging process of centenarians.

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REFERENCES

1. Pawelec G. Immunity and ageing in man. Exp Gerontol 2006;41:1239–1242.
2. Wayne SJ, Rhyne RL, Garry PJ et al. Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. J Gerontol 1990;45:M45–M48.
3. Aspinall R. Longevity and the immune response. Biogerontology 2000;1: 273–278.
4. Guayerbas N, Catalan M, Victor VM et al. Relation of behaviour and macrophage function to life span in a murine model of premature immunosenescence. Behav Brain Res 2002;134:41–48.
5. Guayerbas N, Puerto M, Victor VM et al. Leukocyte function and life span in a murine model of premature immunosenescence. Exp Gerontol 2002;37: 249–256.
6. Guayerbas N, De La Fuente M. An impairment of phagocytic function is linked to a shorter life span in two strains of prematurely aging mice. Dev Comp Immunol 2003;27:339–350.
7. De la Fuente M, Hernanz A, Vallejo MC. The immune system in the oxidative stress conditions of aging and hypertension: Favorable effects of antioxidants and physical exercise. Antioxid Redox Signal 2005;7:1356–1366.
8. Viveros MP, Arranz L, Hernanz A et al. A model of premature aging in mice based on altered stress-related behavioral response and immunosenescence. Neuroimmunomodulation 2007;14:157–162.
9. Puerto M, Guayerbas N, Alvarez P et al. Modulation of neuropeptide Y and norepinephrine on several leucocyte functions in adult, old and very old mice. J Neuroimmunol 2005;165:33–40.
10. Paolisso G, Barbieri M, Bonafe M et al. Metabolic age modelling: The lesson from centenarians. Eur J Clin Invest 2000;30:888–894.
11. Franceschi C, Monti D, Sansoni P et al. The immunology of exceptional individuals: The lesson of centenarians. Immunol Today 1995;16:12–16.

12. Miyaji C, Watanabe H, Toma H et al. Functional alteration of granulocytes, NK cells, and natural killer T cells in centenarians. Hum Immunol 2000;61: 908–916.
13. Vina J, Sastre J, Pallardo FV et al. Role of mitochondrial oxidative stress to explain the different longevity between genders: Protective effect of estrogens. Free Radic Res 2006;40:1359–1365.
14. De la Fuente M, Baeza I, Guayerbas N et al. Changes with ageing in several leukocyte functions of male and female rats. Biogerontology 2004;5:389–400.
15. Butcher SK, Lord JM. Stress responses and innate immunity: Aging as a contributory factor. Aging Cell 2004;3:1511–1560.
16. Niwa Y, Kasama T, Miyachi Y et al. Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: Cross-sectional and longitudinal studies. Life Sci 1989;44:1655–1664.
17. Polignano A, Tortorella C, Venezia A et al. Age-associated changes of neutrophil responsiveness in a human healthy elderly population. Cytobios 1994;80: 145–153.
18. Lord JM, Butcher S, Killampali V et al. Neutrophil ageing and immunesenescence. Mech Ageing Dev 2001;122:1521–1535.
19. De la Fuente M, Hernanz A, Guayerbas N et al. Vitamin E ingestion improves several immune functions in elderly men and women. Free Radic Res 2008; 42:272–280.
20. Meydani SN, Wu D, Santos MS et al. Antioxidants and immune response in aged persons: Overview of present evidence. Am J Clin Nutr 1995;62: 1462S–1476S.
21. de la Fuente M, Hernanz A, Guayerbas N et al. Changes with age in peritoneal macrophage functions. Implication of leukocytes in the oxidative stress of senescence. Cell Mol Biol (Noisy-le-grand) 2004;50 Online Pub:OL683-90.
22. Alvarado C, Alvarez P, Jimenez L et al. Oxidative stress in leukocytes from young prematurely aging mice is reversed by supplementation with biscuits rich in antioxidants. Dev Comp Immunol 2006;30:1168–1180.
23. Paolisso G, Tagliamonte MR, Rizzo MR et al. Oxidative stress and advancing age: Results in healthy centenarians. J Am Geriatr Soc 1998;46:833–838.
24. Barbieri M, Rizzo MR, Manzella D et al. Glucose regulation and oxidative stress in healthy centenarians. Exp Gerontol 2003;38:137–143.
25. Walrand S, Moreau K, Caldefie F et al. Specific and nonspecific immune responses to fasting and refeeding differ in healthy young adult and elderly persons. Am J Clin Nutr 2001;74:670–678.
26. Ligthart GJ, Corberand JX, Fournier C et al. Admission criteria for immunogerontological studies in man: The SENIEUR protocol. Mech Ageing Dev 1984;28:47–55.
27. MacGregor RR, Spagnuolo PJ, Lentnek AL. Inhibition of granulocyte adherence by ethanol, prednisone, and aspirin, measured with an assay system. N Engl J Med 1974;291:642–646.
28. Rodriguez AB, Pariente J, Prieto J et al. Effects of cefmetazol, cefoxitin and imipenem on polymorphonuclear leukocytes. Gen Pharmacol 1987;18: 613–615.
29. De la Fuente M, Carrasco M, Hernanz A. Modulation of human neutrophil function in vitro by gastrin. J Endocrinol 1997;153:475–483.
30. Boyden S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exp Med 1962;115:453–466.
31. De La Fuente M. Changes in the macrophage function with aging. Comp Biochem Physiol A 1985;81:935–938.
32. Bagasra O, Howeedy A, Kajdacsy-Balla A. Macrophage function in chronic experimental alcoholism. I. Modulation of surface receptors and phagocytosis. Immunology 1988;65:405–409.
33. Beers RF Jr., Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 1952;195:133–140.
34. Ferrandez MD, De la Fuente M. Effects of age, sex and physical exercise on the phagocytic process of murine peritoneal macrophages. Acta Physiol Scand 1999;166:47–53.
35. Wenisch C, Patruta S, Daxbock F et al. Effect of age on human neutrophil function. J Leukoc Biol 2000;67:40–45.
36. Esparza B, Sanchez H, Ruiz M et al. Neutrophil function in elderly persons assessed by flow cytometry. Immunol Invest 1996;25:185–190.
37. Alvarez E, Ruiz-Gutierrez V, Sobrino F et al. Age-related changes in membrane lipid composition, fluidity and respiratory burst in rat peritoneal neutrophils. Clin Exp Immunol 2001;124:95–102.
38. Zou Y, Jung KJ, Kim JW et al. Alteration of soluble adhesion molecules during aging and their modulation by calorie restriction. FASEB J 2004;18:320–322.
39. Egger G, Burda A, Mitterhammer H et al. Impaired blood polymorphonuclear leukocyte migration and infection risk in severe trauma. J Infect 2003;47:148–154.
40. Suzuki M, Miura S, Mori M et al. Rebamipide, a novel antiulcer agent, attenuates Helicobacter pylori induced gastric mucosal cell injury associated with neutrophil derived oxidants. Gut 1994;35:1375–1378.
41. Sohal RS, Farmer KJ, Allen RG. Correlates of longevity in two strains of the housefly, *Musca domestica*. Mech Ageing Dev 1987;40:171–179.
42. Sohal RS, Ku HH, Agarwal S. Biochemical correlates of longevity in two closely related rodent species. Biochem Biophys Res Commun 1993;196:7–11.
43. Ali SS, Xiong C, Lucero J et al. Gender differences in free radical homeostasis during aging: Shorter-lived female C57BL6 mice have increased oxidative stress. Aging Cell 2006;5:565–574.
44. Borras C, Gambini J, Vina J. Mitochondrial oxidant generation is involved in determining why females live longer than males. Front Biosci 2007;12: 1008–1013.
45. Vasto S, Candore G, Balistreri CR et al. Inflammatory networks in ageing, age-related diseases and longevity. Mech Ageing Dev 2007;128:83–91.
46. Bhushan M, Cumberbatch M, Dearman RJ et al. Tumour necrosis factor-alpha-induced migration of human Langerhans cells: The influence of ageing. Br J Dermatol 2002;146:32–40.
47. Lang CA, Mills BJ, Lang HL et al. High blood glutathione levels accompany excellent physical and mental health in women ages 60 to 103 years. J Lab Clin Med 2002;140:413–417.
48. Schriner SE, Linford NJ, Martin GM et al. Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 2005;308: 1909–1911.
49. Balbis E, Patriarca S, Furfaro AL et al. Oxidative stress and antioxidant defence in a healthy nonagenarian population. Redox Rep 2007;12:59–62.
50. De Martinis M, Franceschi C, Monti D et al. Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. FEBS Lett 2005;579:2035–2039.