Specific and nonspecific immune responses to fasting and refeeding differ in healthy young adult and elderly persons[1-3]

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ABSTRACT

Background: Undernutrition is a main cause of immunodeficiency. Many confounding factors limit the interpretation of immune function in hospitalized elderly patients.

Objective: We compared the effects of short-term fasting and refeeding on lymphocyte subset distribution and neutrophil function in healthy subjects.

Design: Seven young adult ($\bar{x} \pm SE$ age: 24 ± 2 y) and 8 elderly (71 ± 3 y) subjects were fed standardized diets ($1.6 \times$ predicted resting energy expenditure; 16% protein) for 7 d. They then fasted for 36 h and were refed for 4 h (42 kJ/kg). Lymphocyte subsets were quantified by using fluorochrome-conjugated monoclonal antibodies. Neutrophil chemotactic migration was evaluated by using a 2-compartment chamber. Neutrophil reactive oxygen species production was measured by using a luminol-amplified chemiluminescence assay and oxidation of $2'7'$-dichlorofluorescein diacetate.

Results: Baseline total and cytotoxic T lymphocyte subpopulations were lower in elderly than in adult subjects ($P < 0.01$). Nutritional state had a significant effect ($P < 0.05$) on total, helper, and cytotoxic T and B lymphocyte counts in all subjects, and the response of lymphocyte subpopulations to nutritional fluctuations was significantly affected by age. The chemotactic index was lowered by fasting in both groups ($P < 0.05$ compared with basal values). After refeeding, neutrophil migration was restored in adult but not elderly subjects. The superoxide anion production rate increased with fasting and reverted to prefasting values with refeeding in both groups ($P < 0.05$). Fasting induced a significant decrease in hydrogen peroxide production in stimulated neutrophils that was reversed by refeeding in adult but not elderly subjects.

Conclusion: The lack of response of lymphocyte subpopulation counts and neutrophil function to nutritional changes may help to explain the proneness of elderly persons to infection. *Am J Clin Nutr* 2001;74:670–8.

INTRODUCTION

The decline in immune function with age is unanimously recognized and is supported by many epidemiologic and clinical observations (1–3). These age-related functional changes are responsible for the increased vulnerability to disease of the elderly, which contributes to a higher prevalence of infectious and neoplastic diseases. Furthermore, immune alterations delay recovery after illness. This age-related increase in morbidity will be a major public health concern in the decades to come (4, 5). The most consistent feature of the aging immune system is the heterogeneity of the changes observed. The modifications undergone by each individual component of the immune system are the subject of much controversy. For instance, T suppressor cell populations have been reported to increase (6, 7), decrease (1, 8–10), and remain unchanged (11–15) in elderly subjects. In addition, most studies generally describe a decrease in T helper subsets (1, 10, 12, 13, 16, 17), whereas some investigators detected no variations (6, 8, 9, 14, 15). Likewise, the importance of neutrophils in the immune response has been well documented during aging (18, 19), but divergent data have been reported concerning age-related changes in neutrophil number and function (20–24). The main cause of this marked heterogeneity is the presence of concomitant pathologic disorders that may themselves affect immune status. To overcome this problem, Ligthart et al (25), in the SENIEUR EURAGE protocol, set strict admission criteria for human immunogerontologic studies that include clinical information, laboratory data, and immunopharmacologic interference.

Nutritional status has long been recognized as a major factor in age-related immune impairment, especially in elderly institu-

TABLE 1
Characteristics of subjects[1]

	Adults ($n = 7$)	Elderly ($n = 8$)	Normal range
Age (y)	24 ± 2[2]	71 ± 3	—
Weight (kg)	71.6 ± 2.1	69.9 ± 3.2	—
Height (cm)	173 ± 2	166 ± 3	—
BMI (kg/m^2)	23.5 ± 0.7	25.4 ± 0.7	20–25
Red blood cells			
Total number ($\times 10^9$/L)	5.0 ± 0.2	4.6 ± 0.1	3.8–6.0
Hematocrit	0.447 ± 0.012	0.425 ± 0.005	0.35–0.54
Hemoglobin (mmol/L)	9.5 ± 0.3	8.9 ± 0.1	7.4–11.2
White blood cells ($\times 10^9$/L)	6.8 ± 0.4	6.3 ± 0.4	4–10
Plasma indexes			
Glucose (mmol/L)	5.06 ± 0.28	4.78 ± 0.50	4–6
Insulin (pmol/L)	57.5 ± 8.3	63.1 ± 22.8	50–144
Albumin (g/L)	42 ± 3	38 ± 2	>35
C-reactive protein (mg/L)	<5	<5	<5

[1] There were no significant differences between groups in any of the variables tested.

[2] $\overline{x} \pm$ SEM.

tionalized persons (26–28). Studies in aged, malnourished hospitalized patients have also shown disturbances in cell-mediated and humoral immunity as well as deficiencies in neutrophil function (27–30). However, the interpretation of the results of these studies is hampered by complex environmental influences such as type and degree of illness, cognitive decline, or dementia. Moreover, because immune functions may influence the response to infection and lead to protein wasting and malnutrition, it is difficult to conclude from these studies whether the variations in immunocompetent cell activities were the direct consequence of age, of the disease, or of varying degrees of malnutrition. Thus, the specific interactions between malnutrition and aging on immune status remain poorly understood. Studies devoted to immune modifications during aging should include homogeneous groups of elderly subjects in the same nutritional state and under a controlled nutritional manipulation. It can be hypothesized that immune dysfunction in elderly subjects will be uncovered during nutritional manipulations. The aim of this investigation was thus to study the aging effect by itself (with no disease-related or pharmacologic interference) on immunologic adaptation to nutritional changes. For this purpose, we measured the effect of a 36-h fasting period and of a 4-h refeeding period on specific and nonspecific immune status in healthy elderly and young adult volunteers.

SUBJECTS AND METHODS

Subjects

Seven young adult and 8 elderly subjects took part in the study. Physical and biochemical examinations were performed according to the SENIEUR protocol (25) to select adult and aged subjects in good health (**Table 1**). Briefly, all volunteers were healthy, with no relevant acute or chronic disease that might affect the immune system. In particular, none of the subjects had cancer; serious cardiac, brain, liver, or kidney disease; or any sign of infection or inflammation, and none were taking drugs known to affect the immune system (antiinflammatory drugs, hormones, and analgesics) or had recently been vaccinated.

Our institution is authorized by the French Ministry of Health to perform experiments on healthy subjects. The study was approved by the Ethics Committee of the Auvergne Region. All subjects gave informed, written consent.

Experimental procedure

After a 7-d stabilization period during which the subjects received a standardized diet (1.6 × predicted resting energy expenditure and containing 16% protein) to normalize energy intake, the subjects fasted for 36 h. Under these conditions, glucose is derived mainly from gluconeogenesis, which is highly dependent on amino acid availability. Immune status, which depends on biochemical pathways that are highly dependent on glucose and amino acids, is thereby altered. To avoid dehydration, water intake was carefully controlled so as not to be <1 L/24 h. After this short period of complete starvation, liquid food was reintroduced gradually (50 mL every 30 min) for 4 h (42 kJ/kg per 4-h refeeding period). The refeeding diet contained 18% protein, 32% fat, and 50% carbohydrate. This dietary intake corresponded to one meal consisting of one-third of the daily energy consumption. The diet was supplied in liquid form to optimize gastric emptying and facilitate nutrient absorption. All the subjects were kept under strict medical supervision during the experiment.

Blood sampling

Venous blood samples were collected at the end of the prefasting period (at 0700, 10 h after the last meal) for the measurement of basal values, at the end of the fasting period, and after 4 h of refeeding. Blood was collected into tubes containing EDTA for differential counting (white blood cells and total lymphocyte counts), for measurement of lymphocyte subsets, and for determination of neutrophil function.

Biochemical characteristics

Plasma insulin concentrations were measured by radioimmunoassay (CIS, Gif-sur-Yvette, France). Plasma albumin and C-reactive protein concentrations were measured by immunonephelometry (array protein system; Beckman, Gagny, France) and turbidimetry, respectively, with the use of human antibodies (Dako, Trappes, France).

Assessment of blood cellularity

The total number of leukocytes, leukocyte differential count, red blood cell number, hemoglobin, hematocrit, and platelets were measured with use of a Coulter counter (Coultronics, Margency, France).

Indexes related to specific immune status

Lymphocyte subpopulations were measured by flow cytometry with an Epics Profile (Beckman-Coulter, Villepinte, France) after the blood was prepared with a Q-Prep Epics immunology work station (Beckman-Coulter). Lymphocyte subsets were quantified by immunoreaction with fluorochrome-conjugated monoclonal antibodies by adding different combinations of labeled antibodies to whole blood: CD3-PC$_5$, CD4-RD$_1$, CD8-ECD, CD45-FITC, CD19-ECD, CD16-FITC, and CD56-PE (Beckman-Coulter), where PC is phycoerythrin-cyanin, RD and PE are phycoerythrin, ECD is phycoerythrin Texas red, and FITC is fluorescein isothiocyanate. The panel of CD designations with their appropriate cell names were as follows: CD3$^+$ for total T lymphocytes, CD4$^+$ for T helper-inducer lymphocytes, CD8$^+$

for T suppressor-cytotoxic lymphocytes, CD56+ for immature natural killer lymphocytes, and CD19+ for B lymphocytes.

Intraassay and interassay CVs were recorded by using a lyophilized preparation of human immune leukocytes that exhibited surface antigens (Cyto-Trol Control Cells; Beckman Coulter) and a suspension of fluorospheres that were uniform in size and fluorescence intensity (Flow-Count Fluorospheres; Beckman-Coulter). Intraassay and interassay CVs were <2% for all measurements made with the flow cytometer (lymphocytes subset counts and neutrophil functions). For technical reasons, we could not carry out functional tests to assess lymphocyte activity in this study.

Indexes related to nonspecific immune status

Neutrophil isolation

Six milliliters whole blood was layered onto a discontinuous Ficoll-Hypaque density gradient (Histopaque 1077 and 1119; Sigma, Saint-Quentin-Fallavier, France) and spun (700 × g, 30 min, 20°C). Neutrophils were then collected on the corresponding layer ($1.077 < d < 1.119$) and washed in RPMI-1640 medium (Sigma). Cells were tested for purity (>95%) and viability (>95%) by May-Grunwald-Giemsa staining and the trypan blue dye exclusion test, respectively. The final cell suspension was adjusted in RPMI-1640 medium to the cell density needed for each test by counting in a Malassez chamber (MC2, Clermont-Ferrand, France).

Neutrophil chemotaxis

Freshly isolated neutrophils (1×10^6) were placed in a multiwell insert system containing a 3-μm membrane in a 24-well plate (Becton Dickinson, Meylan, France). Buffer with or without 1×10^{-7} mol/L formyl-Met-Leu-Phe (fMLP; Sigma) was used in the lower chamber to determine chemotaxis and spontaneous migration, respectively. Neutrophils were allowed to migrate for 90 min at 37°C in humidified air containing 5% CO_2 and were then fixed, stained, and counted under a microscope to determine their chemotaxis index. This index corresponds to the ratio of the number of neutrophils that migrated in the lower chamber in response to fMLP to the number of cells that migrated spontaneously (wells without fMLP).

Neutrophil superoxide anion production

Superoxide anion ($O_2^{\bullet-}$) production by neutrophils was measured with a luminol-amplified chemiluminescence assay. Neutrophil suspensions (5×10^5) containing luminol (1 nmol/L; Sigma) were placed in disposable polypropylene vials and the vials were placed in the light-proof chamber of a luminometer (model 1250; LKB Pharmacia, Saint Quentin-en-Yvelines, France) at 37°C. Neutrophils were then stimulated with phorbol 12-myristate 13-acetate (PMA, 1 μmol/L; Sigma) and the resulting light output was continuously recorded on a chart recorder and simultaneously printed out. All results are expressed as mV by using the chemiluminescence emission peak.

Neutrophil hydrogen peroxide production

Hydrogen peroxide production by stimulated neutrophils was measured as previously described (31). Briefly, neutrophils (1×10^6) were preincubated for 15 min with 5 μmol 2'7'-dichlorofluorescin diacetate/L (DCFH-DA; Fluka, Saint-Quentin-Fallavier, France) in a water bath with permanent shaking and temperature control (37°C). Neutrophils were then stimulated with PMA (1 μmol/L). This results in a leukocyte oxidative burst, during which nonfluorescent intracellular DCFH-DA is oxidized to highly fluorescent dichlorofluorescein (DCF) by hydrogen peroxide. DCF fluorescence was recorded by flow cytometry (Beckman-Coulter). Results are expressed as the ratio of hydrogen peroxide produced by PMA-stimulated neutrophils to that produced by unstimulated cells.

Statistical analysis

Data are presented as means ± SEMs and statistical analysis was performed with PCSM software (Deltasoft, Grenoble, France). The experimental design comprised 2 crossed fixed factors with the factor *aging* as 2 classes (adults and elderly) and the factor *nutritional treatment* as 3 classes (control, fasting, and refeeding). When the nutritional treatment effect was significant, we used the letters *C* (control), *F* (fasting), and *R* (refeeding) and the symbols > , <, and = to specify the main effects of these treatments. This design allowed statistical analysis by two-way, repeated-measures analysis of variance (ANOVA). This two-way repeated-measures ANOVA was performed to discriminate between the effects of aging, nutritional treatment, and their interaction. The level of significance was set at $P < 0.05$ for this test. When the ANOVA indicated significant interactions, the Neuman-Keuls test was used to identify differences between individual means. When no significant interaction was found, the Neuman-Keuls test was used to calculate and compare the marginal means. Because the Neuman-Keuls procedure does not control for type I error, we used a Bonferroni correction, for which the significance level was set at $P < 0.02$.

RESULTS

White blood cell count

The total leukocyte population, which includes lymphocytes, monocytes, and neutrophils, was increased by both fasting and refeeding in both groups ($C < F = R$), with no significant effect of age (**Table 2**). This variation was partly due to an increase in neutrophil number during the fasting and the refeeding periods ($C < F = R$).

Indexes related to specific immune status

Nutritional manipulations were associated with a reduction in the lymphocyte proportion ($C > F = R$; Table 2). Additionally, the total lymphocyte count was significantly affected by age and treatment ($C > F = R$).

The percentage of T lymphocytes was significantly altered by the dietary treatment ($C > F < R$; **Table 3**). In addition, the proportion of T lymphocytes was significantly lower in the elderly subjects than in adults. The absolute number of T lymphocytes was affected by nutritional treatment ($C > F = R$), by age, and by an age × nutritional treatment interaction. Specifically, T subsets decreased significantly in adult subjects after the fasting period, and this alteration persisted after refeeding. In addition, these lymphocyte subpopulations were significantly lower during the prefasting, fasting, and refeeding periods in the elderly subjects than in the adults.

An increase in the percentage of B lymphocytes was observed in both groups after refeeding ($C = F < R$; Table 3). Moreover, as shown by the comparison of marginal means with the

TABLE 2
Blood cell indexes in adult and elderly subjects during the control condition (*C*) and after the fasting (*F*) and refeeding (*R*) periods[1]

	Adults			Elderly			
	Control	Fasting	Refeeding	Control	Fasting	Refeeding	ANOVA
Leukocytes ($\times10^9$/L)	6.48 ± 0.51	7.14 ± 0.45	7.57 ± 0.52	5.39 ± 0.55	6.91 ± 0.92	6.85 ± 0.78	*C* < *F* = *R*
Lymphocytes							
(%)	30.7 ± 1.8	24.5 ± 3.2	20.3 ± 1.9	25.8 ± 2.5	20.2 ± 3.5	19.7 ± 2.8	*A*, *C* > *F* = *R*
($\times10^9$/L)	1.98 ± 0.19	1.70 ± 0.21	1.53 ± 0.18	1.32 ± 0.09	1.19 ± 0.11	1.21 ± 0.09	*A*, *C* > *F* = *R*
Monocytes							
(%)	8.36 ± 0.71	7.33 ± 1.01	8.49 ± 0.93	8.85 ± 0.87	7.96 ± 0.48	8.95 ± 0.73	*C* > *F* < *R*
($\times10^9$/L)	0.54 ± 0.05	0.51 ± 0.06	0.63 ± 0.06	0.46 ± 0.04	0.54 ± 0.07	0.63 ± 0.10	*C* = *F* < *R*
Neutrophils							
(%)	57.2 ± 1.7	66.0 ± 3.9	69.5 ± 2.6	60.3 ± 3.9	68.9 ± 4.1	68.9 ± 3.5	*C* < *F* = *R*
($\times10^9$/L)	3.72 ± 0.34	4.78 ± 0.55	5.27 ± 0.43	3.34 ± 0.54	4.97 ± 0.96	4.85 ± 0.79	*C* < *F* = *R*

[1] $\overline{x}$ ± SEM; *n* = 7 or 8 subjects per group. Indexes are expressed both as percentages of the total leukocyte number and as absolute values. Two-way repeated-measures ANOVA was performed to discriminate among the effects of age (*A*; *P* < 0.05), nutritional treatment (*C*, *F*, and *R*; >, significantly more; <, significantly less; =, no significant effect; *P* < 0.05), and their interaction (*I*; *P* < 0.05). A comparison of marginal means with the Bonferroni-corrected Neuman-Keuls test indicated a significant effect of age on lymphocyte subset as a percentage of total leukocytes, *P* < 0.02.

Neuman-Keuls test, a significant age effect was noted. A nutritional effect on the absolute number of B lymphocytes was also observed: the number of B lymphocytes was significantly lowered by the fasting period and corrected by the refeeding regimen (*C* > *F* < *R*). Natural killer cell counts were also significantly affected by age (Table 3).

The percentage of CD4+ cells was reduced after fasting and corrected after refeeding (*C* > *F* < *R*; **Table 4**) in adult and elderly subjects. By contrast, the absolute number of CD4+ cells was reduced after fasting and this alteration persisted during refeeding (*C* > *F* = *R*). The absolute number of CD4+ cells was also significantly affected by age: the T helper lymphocyte subpopulation was significantly lower in the elderly subjects than in the adults. In addition, modifications of T helper subset counts during nutritional manipulations were correlated with variations in albuminemia after fasting and refeeding in the adult subjects (r^2 = 0.24, *P* < 0.05) but not in the elderly ones (data not shown).

In both the adult and elderly subjects, the percentage of CD8+ cells was significantly affected by refeeding (*C* = *F* > *R*; Table 4). The absolute number of cytotoxic T lymphocytes was signifi-

cantly affected by dietary treatment (*C* < *F* < *R*), age, and their interaction: the absolute number of CD8+ cells was lower during fasting and refeeding than during the control period in adult subjects and was lower in the elderly subjects than in the adults during all treatments. These changes in the cytotoxic T lymphocytes were correlated with variations in albuminemia induced by the dietary treatments (data not shown) in the elderly group (r^2 = 0.77, *P* < 0.01). Because both the CD4+ and the CD8+ populations were affected by nutritional treatment, the ratio of CD4+ to CD8+ cells was lower in fasted subjects (*C* > *F* < *R*; Table 4).

Indexes related to nonspecific immune function

Neutrophil chemotaxis

During fasting, the neutrophil chemotaxis index was lower than basal control values in adult and elderly subjects (**Figure 1**). Refeeding induced a significant enhancement of neutrophil migration responsiveness in the adults. In addition, a correlation between the chemotaxis index and variations in glycemia induced by nutritional manipulations (data not shown) was found only in

TABLE 3
Lymphocyte distribution in adult and elderly subjects during the control condition (*C*) and after the fasting (*F*) and refeeding (*R*) periods[1]

	Adults			Elderly			
	Control	Fasting	Refeeding	Control	Fasting	Refeeding	ANOVA
T lymphocytes							
(%)	78.1 ± 1.8	74.5 ± 1.8	75.7 ± 2.1	70.8 ± 3.9	67.8 ± 3.0	70.0 ± 3.2	*A*, *C* > *F* < *R*
($\times10^9$/L)	1.54 ± 0.15	1.28 ± 0.18[2]	1.16 ± 0.14[2]	0.94 ± 0.08[3]	0.82 ± 0.10[3]	0.85 ± 0.08[3]	*A*, *C* > *F* = *R*, *I*
B lymphocytes							
(%)	12.1 ± 0.8	12.3 ± 1.5	14.3 ± 1.6	16.1 ± 2.6	16.2 ± 2.6	18.3 ± 2.9	*A*, *C* = *F* < *R*
($\times10^9$/L)	0.24 ± 0.03	0.19 ± 0.02	0.21 ± 0.03	0.21 ± 0.03	0.20 ± 0.04	0.22 ± 0.04	*C* > *F* < *R*
NK lymphocytes							
(%)	5.8 ± 1.3	6.4 ± 1.6	3.1 ± 1.1	1.4 ± 0.4	4.0 ± 1.8	2.7 ± 1.4	NS
($\times10^9$/L)	0.11 ± 0.03	0.11 ± 0.03	0.05 ± 0.02	0.02 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	*A*

[1] $\overline{x}$ ± SEM; *n* = 7 or 8 subjects per group. Lymphocytes are expressed both as percentages of the total lymphocyte number and as absolute values. Two-way repeated-measures ANOVA was performed to discriminate among the effects of age (*A*; *P* < 0.05), nutritional treatment (*C*, *F*, and *R*; >, significantly more; <, significantly less; =, no significant effect; *P* < 0.05), and their interaction (*I*; *P* < 0.05). When no significant interaction was noted, a comparison of marginal means was carried out with the Bonferroni-corrected Neuman-Keuls test, which indicated a significant effect of age on T and B lymphocyte subsets as a percentage of total lymphocytes, *P* < 0.02. Because a significant interaction was found for the absolute number of T lymphocytes, comparison of individual means was valid and was carried out with the Bonferroni-corrected Neuman-Keuls test. NK, natural killer.

[2] Significantly different from the control adult group, *P* < 0.02.

[3] Significantly different from adults, *P* < 0.02.

TABLE 4
T lymphocyte distribution in adult and elderly subjects during the control condition (C) and after the fasting (F) and refeeding (R) periods[1]

| | Adults | | | Elderly | | | |
	Control	Fasting	Refeeding	Control	Fasting	Refeeding	ANOVA
CD4+ cells							
(%)	50.7 ± 3.1	48.0 ± 2.7	50.9 ± 3.0	51.5 ± 3.1	47.6 ± 2.8	51.5 ± 3.2	$C > F < R$
(×10⁹/L)	1.00 ± 0.10	0.83 ± 0.12	0.79 ± 0.11	0.68 ± 0.06	0.57 ± 0.06	0.62 ± 0.06	$A, C > F = R$
CD8+ cells							
(%)	27.8 ± 1.8	28.4 ± 1.9	25.6 ± 1.9	23.1 ± 2.5	24.3 ± 2.9	22.5 ± 2.7	$C = F > R$
(×10⁹/L)	0.56 ± 0.07	0.48 ± 0.07[2]	0.39 ± 0.05[2,3]	0.31 ± 0.04[4]	0.29 ± 0.05[4]	0.27 ± 0.04[4]	$A, C < F < R, I$
CD4+:CD8+	1.91 ± 0.23	1.76 ± 0.20	2.09 ± 0.25	2.48 ± 0.88	2.22 ± 0.33	2.65 ± 0.47	$C > F < R$

[1] $\bar{x}$ ± SEM; n = 7 or 8 subjects per group. Lymphocytes are expressed both as percentages of the total CD3+ lymphocyte number and as absolute values. Two-way repeated-measures ANOVA was performed to discriminate among the effects of age (A; $P < 0.05$), nutritional treatment (C, F, and R; >, significantly more; <, significantly less; = , no significant effect; $P < 0.05$), and their interaction (I; $P < 0.05$). When no significant interaction was noted, a comparison of marginal means was carried out with the Bonferroni-corrected Neuman-Keuls test, which indicated a significant effect of age on CD4+:CD8+, $P < 0.02$. Because a significant interaction was found for the absolute number of CD8+ cells, comparison of individual means was valid and was carried out with the Bonferroni-corrected Neuman-Keuls test.
[2] Significantly different from the control adult group, $P < 0.02$.
[3] Significantly different from the fasting adult group, $P < 0.02$.
[4] Significantly different from adults, $P < 0.02$.

adults (r^2 = 0.38, $P < 0.01$). By contrast, refeeding had no beneficial effect on neutrophil chemotaxis in the elderly subjects.

Neutrophil superoxide anion production

Fasting resulted in a significant increase in $O_2^{\bullet-}$ production in both the adult and elderly subjects ($C < F > R$; **Figure 2**). Refeeding led to a recovery of basal $O_2^{\bullet-}$ generation by stimulated neutrophils whatever the subject's age.

Neutrophil hydrogen peroxide production

Nutritional modifications were characterized by alterations in hydrogen peroxide generation by PMA-activated neutrophils in adult and elderly subjects ($C > F = R$; **Figure 3**).

DISCUSSION

We hypothesized that subtle or obvious alterations in immune function might precede the development of disease in elderly subjects, and that nutritional state might be a major factor in a limited immune response even before hospitalization. To test this hypothesis, we chose a population free of pathologic disorders according to the SENIEUR protocol. This stringent selection is crucial because many confounding factors such as inflammation, sepsis, cancer, and ischemic heart disease modify immune status. We found marked differences between adult and elderly healthy individuals in both the number of some lymphocyte subsets and neutrophil functions. The results of our study show that age by itself affected not only basal immune function but also the response to various nutritional states, implying that nutritional state is an important consideration in the clinical management of elderly patients.

As previously described (1), our results indicate that basal counts of total T lymphocytes and the cytotoxic T lymphocyte subset were lower in the elderly than in the adult subjects. Because we selected only healthy subjects unimpaired by any illness and taking no medication liable to exert immunomodulating effects, we can postulate various underlying mechanisms,
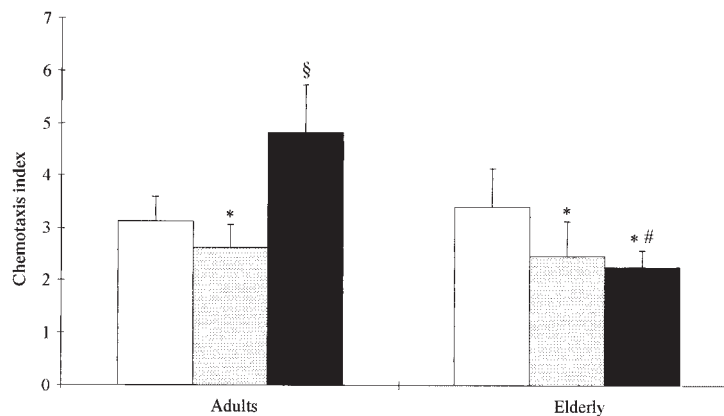

FIGURE 1. Mean (±SEM) neutrophil chemotaxis index (ratio of the number of neutrophils that migrated in response to formyl-Met-Leu-Phe to the number that migrated spontaneously) in healthy adult (n = 7) and elderly (n = 8) subjects. The subjects were fed a standardized diet for 7 d (control; □), fasted for 36 h (▨), and were then refed with a liquid diet introduced gradually over 4 h (42 kJ/kg; ■). There was a significant age × nutritional treatment interaction, $P < 0.05$ (two-way repeated-measures ANOVA). Individual means were compared by using the Bonferroni-corrected Neuman-Keuls test: *Significantly different from the respective control treatment, $P < 0.02$; §Significantly different from fasting in adults, $P < 0.02$; #Significantly different from refeeding in adults, $P < 0.02$.
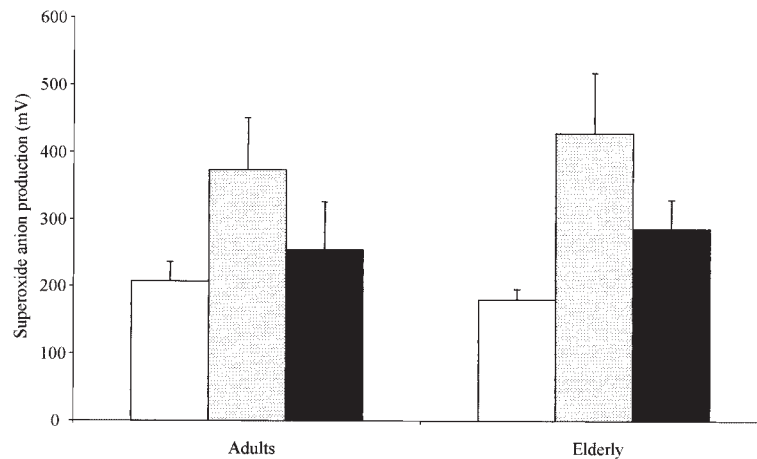
FIGURE 2. Mean (±SEM) superoxide anion production by phorbol 12-myristate 13-acetate–stimulated neutrophils in healthy adult ($n = 7$) and elderly ($n = 8$) subjects. The subjects were fed a standardized diet for 7 d (control; □), fasted for 36 h (▨), and were then refed with a liquid diet introduced gradually over 4 h (42 kJ/kg; ■). There was a significant effect of nutritional treatment (control < fasting > refeeding), $P < 0.05$ (two-way repeated-measures ANOVA).

especially for the antigen expression of lymphocytes, which appears to be affected by aging itself. First, age-related changes in the thymus tissue may explain the decline in T cell–dependent immunity (32). Nevertheless, because the thymus undergoes involution before the onset of age-related changes in immune function, the thymus may be only partly responsible for the changes in T lymphocytes with age. Another explanation may be antigen expression on T lymphocyte subsets, which is subject to qualitative and quantitative modifications with advancing age (13, 33, 34). The CD25 antigen, which is identified as the α chain of the high-affinity interleukin 2 receptor, decreased with age in both helper and cytotoxic lymphocytes (13), making interleukin 2 less effective for lymphocyte proliferation (8, 12). Conversely, a marked $CD3^+HLA\text{-}DR^+$ up-regulation was described with increasing age, suggesting an increase in antigen presentation by T lymphocytes (6). Such qualitative and quantitative age-related changes in antigen expression on T lymphocytes may

have a major influence on cell activation and functions during adaptative challenges (infectious diseases, inflammation, trauma, and protein-energy malnutrition). In addition, T helper lymphocytes have a specific role in helping B lymphocytes to synthesize antibodies, so that a reduction in the number of these cells favors a decrease in humoral response capacity (12, 35).

For natural killer lymphocytes, we showed a significant decline in the $CD3^-CD16^+CD56^+$ phenotype (immature cells) with age. Such an immunosenescent change in the immature natural killer cell subset is a potential immunologic disadvantage for elderly subjects because these lymphocytes have high cytotoxic potential (36, 37). Nevertheless, Krishnaraj et al (38, 39) reported that the decrease in immature natural killer cells was accompanied by an up-regulation of $CD3^-CD16^+CD57^+$ mature natural killer cells in elderly persons.

Because baseline changes in immune status occur in elderly subjects, it is of major interest to determine the influence of
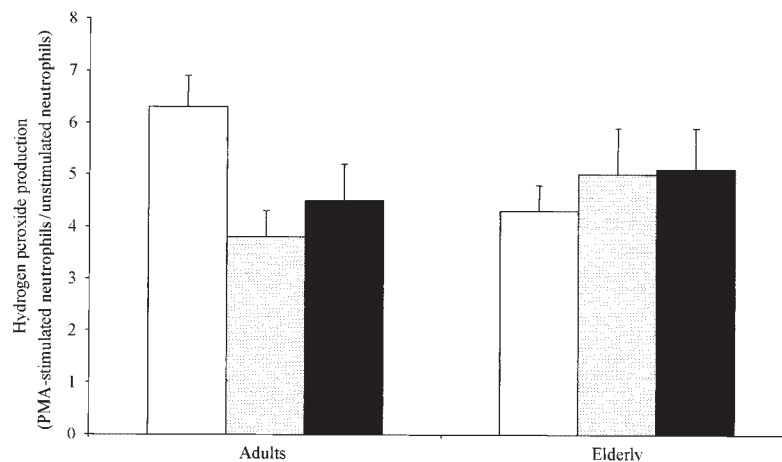

FIGURE 3. Mean (±SEM) hydrogen peroxide production by phorbol 12-myristate 13-acetate (PMA)–stimulated neutrophils in healthy adult ($n = 7$) and elderly ($n = 8$) subjects. The subjects were fed a standardized diet for 7 d (control; □), fasted for 36 h (▨), and were then refed with a liquid diet introduced gradually over 4 h (42 kJ/kg; ■). There was a significant effect of nutritional treatment (control > fasting = refeeding), $P < 0.05$ (two-way repeated-measures ANOVA).

nutritional intervention on the immune response in this population. In adults, fasting is known to cause rapid changes in immune markers similar to those that occur during anorexia nervosa (40). In our study, the total white blood cell count increased gradually, whereas the total and subpopulation numbers of lymphocytes decreased significantly during fasting in adults. These findings contrast with the observation that blood cell variables did not change significantly after starvation in obese subjects (41, 42). The discrepancies observed in subjects of different nutritional status point to the necessity of undertaking a detailed immunologic investigation in these patients.

The decrease in T lymphocyte counts in adult fasted subjects was stabilized by food intake, except for cytotoxic T lymphocytes. Nevertheless, the marked difference between the durations of starvation and refeeding, particularly the limited length of the refeeding period, may explain the slight modifications observed after food restitution. The postprandial decrease in cytotoxic lymphocytes probably reflects cell migration into extravascular lymphoid tissues, particularly at the intestinal level. In this tissue, suppressor T lymphocytes may serve to support primary local immune response against antigen exposure, which is enhanced by food intake (43). In the same way, although no modification in B lymphocytes was observed during fasting, the increase in B lymphocytes described after the refeeding period promotes immunoglobulin production and contributes to the induction of immune function after meal absorption (44).

The neutrophil functional tests that were used are representative of the key antimicrobial functions of these cells. Neutrophil migration explores a basic property of neutrophils required for contacting and ultimately destroying invading organisms (45). Bacterial cytotoxicity results from the action of oxygen species, eg, $O_2^{\bullet-}$ and hydrogen peroxide, that are the major neutrophil microbicidal agents (18). According to our results, the basal functional indexes of aged neutrophils were barely affected in vitro. Some authors (22, 24) showed a significant decline in chemotactic responsiveness and bactericidal activity in aged persons, whereas others (20, 23) did not. These discrepancies may be partially ascribed to the population tested and the techniques used.

Acute fasting decreased neutrophil chemoattractant ability in both the adult and elderly healthy subjects. Chemotaxis is a highly glucose-dependent biochemical pathway (42, 46) and the reduced glucose availability during fasting, together with hypoinsulinemia, may limit glucose uptake and utilization by neutrophils. This last observation might help to explain the recovery of chemotaxis capacity by adult neutrophils after refeeding and the correlation found between the chemotaxis index and glycemia variations. Nevertheless, a significantly lower chemotactic response of neutrophils to refeeding was observed in the healthy elderly persons than in the younger adults. The mechanism of this alteration is not known, but potential targets for age-related dysfunction in chemotaxis include the cytoskeleton (47), the cell membrane (48), surface receptors (47), transduction mechanisms (49, 50), cyclic nucleotide metabolism (51), and glucose utilization (46). The effect of aging and nutritional stress on each of these areas will now need to be determined.

Fasting clearly induced an increase in $O_2^{\bullet-}$ generation. The oxidative burst depends on the activity of NADPH,H^+ oxidase, a transmembrane electron transport chain that reduces oxygen to $O_2^{\bullet-}$ (52). When neutrophils are activated by a stimulant, PMA, the multicomponent system of NADPH,H^+ oxidase, is rapidly assembled and activated (21). PMA activates protein kinase C (18), which is involved in the activation of NADPH,H^+ oxidase (18). Some authors have reported that fasting is accompanied by a marked increase in protein kinase C activity, resulting in stimulation of NADPH,H^+ oxidase activity (53, 54). This last observation may then explain the increase in $O_2^{\bullet-}$ production we noted during fasting. In addition, $O_2^{\bullet-}$ dismutation leads to the formation of hydrogen peroxide, which participates in the microbicidal action of neutrophils (18). Some differences in hydrogen peroxide generation between the adult and elderly persons in our study were observed during starvation. As previously described (29, 55, 56), elderly individuals under nutritional stress exhibit metabolic pathway imbalances in neutrophil hydrogen peroxide scavenging enzymes, resulting in a decrease in total cellular myeloperoxidase, catalase, and glutathione peroxidase. The dysregulation of the reactive oxygen species generation pathway during aging may explain the differences in hydrogen peroxide generation noted in this study after starvation. After the refeeding period, $O_2^{\bullet-}$ production by neutrophils from the adult and elderly subjects decreased and returned to normal values. These last results imply that NADPH,H^+ oxidase displays a high sensitivity to nutritional variations. We also showed that elderly persons do not adapt to nutritional repletion, presenting a high rate of hydrogen peroxide production despite a decrease in $O_2^{\bullet-}$ generation during refeeding.

In conclusion, despite the limited number of subjects in this study, we showed that short-term fasting and refeeding affect several immunologic indexes in elderly persons, in whom aging already favors intrinsic changes in immunity. The mechanisms of the impairment in the ability of lymphocytes and neutrophils to adapt to nutritional manipulations are still a matter of speculation and may involve either an intrinsic defect in the immune cells that exists before the cells are released into the bloodstream or an acquired defect related to environmental components depending on both the aging process and nutritional status. These findings highlight the specific effects of age and nutritional state on immune function and suggest that age is a risk factor for reduced immunity, even in a healthy population. A superimposed infection or inflammation will presumably amplify these alterations. Finally, besides age and pathologic disorders, undernutrition has an adverse effect on immune function. This finding needs to be taken into account in the care of vulnerable elderly hospitalized patients. In this population, protein-energy supply or even specific immunomodulating nutritional agents must be carefully administered to aid the recovery of immune function and limit the consequences of infectious challenge.

REFERENCES

1. Huppert FA, Solomou W, O'Connor S, Morgan K, Sussams P, Brayne C. Aging and lymphocyte subpopulations: whole-blood analysis of immune markers in a large population sample of healthy elderly individuals. Exp Gerontol 1998;33:593–600.
2. Lesourd BM, Meaume S. Cell mediated immunity changes in ageing, relative importance of cell subpopulation switches and of nutritional factors. Immunol Lett 1994;40:235–42.

3. Chandra RK. Nutritional regulation of immunity and risk of infection in old age. Immunology 1989;67:141–7.

4. Vellas BJ, Albarede JL, Garry PJ. Diseases and aging: patterns of morbidity with age; relationship between aging and age-associated diseases. Am J Clin Nutr 1992;55(suppl):1225S–30S.

5. 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;45:45–8.

6. Stulnig T, Maczek C, Böck G, Majdic O, Wick G. Reference intervals for human peripheral blood lymphocyte subpopulations from "healthy" young and aged subjects. Int Arch Allergy Immunol 1995;108:205–10.

7. Bunker VW, Stansfield MF, Deacon-Smith R, Marzil RA, Hounslow A, Clayton BE. Dietary supplementation and immunocompetence in housebound elderly subjects. Br J Biomed Sci 1994;51:128–35.

8. Sindermann J, Kruse A, Frercks H, Schütz RM, Kirchner H. Investigations of the lymphocyte system in elderly individuals. Mech Ageing Dev 1993;70:149–59.

9. Nagel JE, Chrest FJ, Adler WH. Enumeration of T lymphocyte subsets by monoclonal antibodies in young and aged humans. J Immunol 1981;127:2086–8.

10. Sansoni P, Cossarizza A, Brianti V, et al. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. Blood 1993;82:2767–73.

11. Arnalich F, Hernanz A, Vàzquez JJ, Amores A. Cell-mediated immune response and cytokine production in idiopathic senile anorexia. Mech Ageing Dev 1994;77:67–74.

12. Rea IM, Alexander HD, Crockard AD, Morris TCM. CD4 lymphopenia in very elderly people. Lancet 1996;347:328–9.

13. Rea IM, McNerlan SE, Alexander HD. CD69, CD25, and HLA-DR activation antigen expression on CD3+ lymphocytes and relationship to serum TNF-α, INF-γ, and sIL2R levels in aging. Exp Gerontol 1999;34:79–93.

14. Matour D, Melnicoff M, Kaye D, Murasko DM. The role of T cell phenotypes in decreased lymphoproliferation of the elderly. Clin Immunol Immunopathol 1989;50:82–99.

15. Hallgren HM, Jackola DR, O'Leary JJ. Unusual pattern of surface marker expression on peripheral lymphocytes from aged humans suggestive of a population of less differentiated cells. J Immunol 1983;131:191–4.

16. Gianni W, Cacciafesta M, Vetta F, Marigliano V, Ippoliti F. Nutrition and immunity in older people. J Am Geriatr Soc 1997;45:1538–9.

17. Rea IM, Stewart M, Campbell P, Alexander HD, Crockard AD, Morris TCM. Changes in lymphocyte subsets, interleukin 2, and soluble interleukin 2 receptor in old and very old age. Gerontology 1996;42:69–78.

18. Casimir CM, Teahan CG. The respiratory burst of neutrophils and its deficiency. In: Hellewell PG, Williams TJ, eds. Immunopharmacology of neutrophils. San Diego: Academic Press, 1994:27–54.

19. Doherty NS, Janusz MJ. Neutrophil proteases: their physiological and pathological roles. In: Hellewell PG, Williams TJ, eds. Immunopharmacology of neutrophils. San Diego: Academic Press, 1994:55–94.

20. Fietta A, Merlini C, Dos Santos C, Rovida S, Grassi C. Influence of aging on some specific and nonspecific mechanisms of the host defense system in 146 healthy subjects. Gerontology 1994;40:237–45.

21. Braga PC, Sala MT, Dal Sasso M, Pecile M, Annoni G, Vergani C. Age-associated differences in neutrophil oxidative burst (chemiluminescence). Exp Gerontol 1998;33:477–84.

22. Biasi D, Carletto A, Dell'Agnola C, et al. Neutrophil migration, oxidative metabolism, and adhesion in elderly and young subjects. Inflammation 1996;20:673–81.

23. Kuroiwa A, Miyamoto K, Okabe N, Shibuya T. Re-evaluation of the phagocytic respiratory burst in the physiological or inflammatory state and in ageing. J Clin Lab Immunol 1989;29:189–91.

24. Chan SS, Monteiro HP, Deucher GP, Abud RL, Abuchalla D, Junqueira VB. Functional activity of blood polymorphonuclear leukocytes as an oxidative stress biomarker in human subjects. Free Radic Biol Med 1998;24:1411–8.

25. 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.

26. Incalzi RA, Capparella O, Gemma A, et al. Inadequate caloric intake: a risk factor for mortality of geriatric patients in the acute-care hospital. Age Ageing 1998;27:303–10.

27. Chandra RK. Nutrition is an important determinant of immunity in old age. Prog Clin Biol Res 1990;326:321–34.

28. Lesourd B. Protein undernutrition as the major cause of decreased immune function in the elderly: clinical and functional implications. Nutr Rev 1995;53:S86–94.

29. Antonaci S, Jirillo E, Polignano A, Tortorella C, Bonomo L. Phagocyte dysfunctions in malnourished elderly humans: effects of in vitro nutrient supplementation. Nutr Res 1991;11:875–84.

30. Cederholm T, Gyllenhammar H. Impaired granulocyte formylpeptide-induced superoxide generation in chronically ill, malnourished, elderly patients. J Intern Med 1999;245:475–82.

31. Walrand S, Chambon-Savanovitch C, Felgines C, et al. Aging: a barrier to renutrition? Nutritional and immunologic evidence in rat. Am J Clin Nutr 2000;72:816–24.

32. James SJ, Castle SC, Makinodan T. Decline in immune function with age: interaction with specific nutrient deficiencies. In: Morley JE, Glick Z, Rubenstein LZ, eds. Geriatric nutrition. New York: Raven Press, 1995:153–67.

33. Wick G, Grubeck-Loebenstein B. The aging immune system: primary and secondary alterations of immune reactivity in the elderly. Exp Gerontol 1997;32:401–13.

34. Shearer GM. Th1/Th2 changes in aging. Mech Ageing Dev 1997; 94:1–5.

35. Paganelli R, Quinti I, Fagiolo U, et al. Changes in circulating B cells and immunoglobulin classes and subclasses in a healthy aged population. Clin Exp Immunol 1992;90:351–4.

36. Ogata K, Yokose N, Tamura H, et al. Natural killer cells in the late decades of human life. Clin Immunol Immunopathol 1997;84:269–75.

37. McNerlan SE, Rea IM, Alexander HD, Morris TCM. Changes in natural killer cells, the CD57CD8 subset, and related cytokines in healthy aging. J Clin Immunol 1998;18:31–8.

38. Krishnaraj R, Svanborg A. Preferential accumulation of mature NK cells during human immunosenescence. J Cell Biochem 1992;50: 386–91.

39. Krishnaraj R, Blandford G. Age-associated alterations in human natural killer cells. Clin Immunol Immunopathol 1987;45:268–85.

40. Marcos A, Varela P, Toro O, et al. Interactions between nutrition and immunity in anorexia nervosa: a 1-y follow-up study. Am J Clin Nutr 1997;66(suppl):485S–90S.

41. Wing EJ, Stanko RT, Winkelstein A, Adibi SA. Fasting-enhanced immune effector mechanisms in obese subjects. Am J Med 1983;75: 91–6.

42. McMurray RW, Bradsher RW, Steele RW, Pilkington NS. Effect of prolonged modified fasting in obese persons on in vitro markers of immunity: lymphocyte function and serum effects on normal neutrophils. Am J Med Sci 1990;299:379–85.

43. Komaki G, Kanazawa F, Sogawa H, et al. Alterations in lymphocyte subsets and pituitary-adrenal gland-related hormones during fasting. Am J Clin Nutr 1997;66:147–52.

44. Chandra RK. Food hypersensitivity and allergic disease: a selective review. Am J Clin Nutr 1997;66(suppl):526S–9S.

45. Rossi AG, Hellewell PG. Mechanisms of neutrophil accumulation in tissues. In: Hellewell PG, Williams TJ, eds. Immunopharmacology of neutrophils. San Diego: Academic Press, 1994:223–43.

46. Mowat AG, Baum J. Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. N Engl J Med 1971;284: 621–7.

47. Rao KMK, Currie MS, Padmanabhan J, Cohen HJ. Age-related alterations in actin cytoskeleton and receptor expression in human leukocytes. J Gerontol 1992;47:37–44.

48. Rivnay B, Bergman S, Shinitzky M, Globerson A. Correlation between membrane viscosity, serum cholesterol, lymphocyte activation and aging in man. Mech Ageing Dev 1980;12:119–26.

49. Lipschitz DA, Udupa KB, Boxer LA. The role of calcium in the age-related decline of neutrophil function. Blood 1988;71:659–65.

50. Lipschitz DA, Udupa KB, Indelicato SR, Das M. Effect of age on second messenger generation in neutrophils. Blood 1991;78:1347–54.

51. Hatch GE, Nichols WK, Hill HR. Cyclic nucleotide changes in human neutrophils induced by chemoattractants and chemotactic modulators. J Immunol 1977;119:450–6.

52. Akard LP, English D, Gabig TG. Rapid deactivation of NADPH oxidase in neutrophils: continuous replacement by newly activated enzyme sustains the respiratory burst. Blood 1988;72:322–7.

53. Pineiro V, Casabiell X, Peino R, Garcia-Vallejo L, Dieguez C, Casanueva FF. PMA inhibits both spontaneous and glucocorticoid-mediated leptin secretion by human omental adipose tissue explants in vitro. Biochem Biophys Res Commun 1998;252:345–7.

54. Karasik A, Rothenberg PL, Yamada K, White MF, Kahn CR. Increased protein kinase C activity is linked to reduced insulin receptor autophosphorylation in liver of starved rats. J Biol Chem 1990;265:10226–31.

55. Niwa Y, Iizawa O, Ishimoto K, Akamatsu H, Kanoh T. Age-dependent basal level and induction capacity of copper-zinc and manganese superoxide dismutase and other scavenging enzyme activities in leukocytes from young and elderly adults. Am J Pathol 1993;143:312–20.

56. Tortorella C, Ottolenghi A, Pugliese P, Jirillo E, Antonaci S. Relationship between respiratory burst and adhesiveness capacity in elderly polymorphonuclear cells. Mech Ageing Dev 1993;69:53–63.