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Relationships Between Lifestyle Factors and Neutrophil Functions in the Elderly

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We investigated the relationships between neutrophil functions and lifestyle factors in the elderly. The subjects (84 males, 73.9±5.8 years old; and 63 females, 70.0±4.6 years old) belonged to a recreational seniors club in Japan. Investigations of the subjects' stress, exercise habits, smoking habits, and alcohol-drinking habits were performed. The phagocytosis and superoxide productivity of the neutrophils were measured with a nitroblue tetrazolium (NBT) reduction test. In addition, leukocyte counts and serum total protein (TP) levels were determined. The results revealed that aging, high serum levels, and stress-coping factors (e.g., having hobbies, keeping pets, and close links with friends or family) significantly correlated with preferable neutrophil functions. In addition, significant effects of lifestyle factors on the balance between phagocytosis and subsequent superoxide production were observed. Thus, the results of the present study suggest that there are correlations between neutrophil functions and lifestyle factors in the elderly. J. Clin. Lab. Anal. 16:266–272, 2002.
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INTRODUCTION

Elderly people are apt to be affected by infections, and such an infection, once established, tends to become intractable and carry a high risk of mortality (1). This has been attributed to decreased immune and physiological functions as a consequence of aging (2). Since the neutrophils play an important role in initial protection against infections (3), it is thought that decreased neutrophil function leads to susceptibility to infection in the elderly. It has been reported that neutrophil function in the elderly is significantly lower than that in adults (4).

The relationship between immune function and lifestyle has also been studied (5,6), and some reports have indicated that neutrophil function is influenced by stress (7–9), training activity (10), and state of nutrition (11). It has been suggested that unfavorable lifestyles in the elderly may decrease the neutrophil function and cause infectious diseases. However, few studies have investigated the relationship between neutrophil function and lifestyle in the elderly. In particular, very few investigations have been conducted on the relationship between neutrophil function and psychological stress in the elderly. From the viewpoint of preventive medicine, it is important to investigate such relationships.

In the present study, we examined neutrophil function with a nitroblue tetrazolium (NBT) reduction test (12,13), which enables the simultaneous measurement of phagocytosis and superoxide production. This technique can determine the functions in a condition close to those in an in vivo environment because the neutrophils are not isolated from the whole blood. In our previous study (14) using mice and rats, we reported

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that the positive correlation between phagocytosis and superoxide productivity was influenced by exercise and stress, respectively, suggesting that the correlations between the phagocytic activity and superoxide production by the neutrophils reflect whether or not the neutrophil functional state is appropriately controlled. However, these phenomena have not yet been investigated in humans. In this study, therefore, we investigated epidemiologically the relationships among neutrophil functions, lifestyle factors (stress, smoking, alcohol intake, exercise habit, and nutritional intake), and age in the elderly.

MATERIALS AND METHODS

Subjects

The subjects were elderly people (more than 60 years of age) who belonged to a recreational seniors club in Sayama City, Saitama Prefecture, Japan. They participated in the study voluntarily. The subjects included 84 males (73.9 ± 5.8 years old) and 63 females (70.0 ± 4.6 years old). Those who were affected by common cold on the day of the survey were excluded from the study.

Evaluation of Stress, Exercise, Smoking, and Alcohol-Drinking Habits

The degree of subjective stress was examined using a simplified stress checklist (SCL-S) developed by Katsura et al. (15). A score of 10 or less indicates mild stress, 11–20 indicates moderate stress, and 21 or more indicates severe stress. Concerning the treatment of stress, the subjects were asked about the presence or absence of stress-coping factors against the affliction. Regarding the stress-coping factors, we asked about boarding with families, the presence of intimate associates, keeping of pets, having a hobby or religion, and smoking and alcohol-drinking habits. We also inquired about exercise habits. We defined the subjects who exercised for 1 hr or more per week as the exercise group.

Serum Biochemical Analysis

Peripheral venous blood samples were obtained by antecubital venipuncture in the early morning after overnight fasting. After the blood was allowed to clot at room temperature, the serum samples were separated by centrifugation at 1,000 *g* for 10 min to determine the total protein (TP) as an index of nutritional state. The TP was measured with biochemical assay kits prescribed for the Clinical Chemistry Analyzer CL-7000 (Shimadzu Co., Ltd., Tokyo, Japan).

Assay of Neutrophil Functions

Phagocytosis and superoxide productivity by the neutrophils were measured with the NBT reduction test. *Staphylococcus aureus* 209P was used as the foreign agent. Phagocytosis was determined from the number of ingested particles per cell. Superoxide production was evaluated by the classification of each cell into four types (0, I, II, and III) depending on the relative extent of the NBT reduction to blue-black formazan, and was scored by calculating the square of the type number for the degree of the NBT reduction (13). The NBT reduction score was expressed as the average score per cell.

Statistical Analysis

The values were expressed as the mean $\pm$ SD. A *t*-test was used for comparison of the mean values. A comparison of frequency was performed using Fisher's exact probability test. Pearson's correlation coefficient (r) was used for analysis of correlation. Furthermore, multiple regression analysis was performed using the neutrophil functions as dependent variables for calculation of the partial regression coefficient (β) while assigning age, stress, exercise, smoking, and alcohol-drinking habits, total leukocyte count, and serum TP level as independent variables. *P*-values less than 0.05 were considered significant.

RESULTS

Gender Difference in Examined Items

The results of gender difference in the examined items are shown in Table 1. Significant differences were found in ages ($P < 0.01$), number of subjects who had a smoking habit ($P < 0.01$) and alcohol-drinking habit ($P < 0.01$), neutrophil NBT reduction activity ($P < 0.05$), and TP ($P < 0.01$). Tobacco consumption was 18.3 ± 9.5 cigarettes per day in the males ($n = 26$), and 13.0 ± 6.7 cigarettes per day in the females ($n = 5$). The alcohol intake by the drinkers was 26.4 ± 5.1 g per day in the males ($n = 54$), and 11.1 ± 3.2 g per day in the females ($n = 20$). No significant differences were observed in the state of subjective stress and stress-coping factors between male and female subjects.

Relationships Between Lifestyle Factors and Neutrophil Functions

The relationships between neutrophil function and age, subjective stress score, neutrophil count, and TP are shown in Table 2. In all of the subjects, a significant correlation between phagocytosis and TP was observed ($r = 0.229$, $P < 0.01$), and significant correlations between NBT reduction activity and age ($r = 0.219$,

TABLE 1. Comparison between males and females

	Total ($n=147$)	Males ($n=84$)	Females ($n=63$)
Age (years)	72.2±5.5	73.9±5.8	70.0±4.6**
Subjective stress score	9.5±7.0	9.5±6.5	9.5±7.6
Stress-coping factor (≧1)	48.3% (761/145)	46.4% (39/82)	50.7% (32/63)
Exercise habit (≧1 h/week)	73.5% (108/147)	72/6% (61/84)	74.6% (47/63)
Smoking habit (current smoker)	21.1% (31/147)	30.9% (26/84)	7.9% (5/63)**
Alcohol-drinking habit (yes)	50.3% (74/147)	64.3% (54/84)	31.7% (20/63)**
Total leukocyte count (10^3/mm^3)	5.7±1.3	5.8±1.3	5.6±1.3
Phagocytosis (particle/cell)	8.2±3.0	8.0±3.2	8.4±2.8
NBT reduction activity (score/cell)	1.0±0.5	1.1±0.5	0.9±0.5*
TP (g/dl)	7.1±0.5	7.0±0.5	7.3±0.5**

Values indicate mean±SD.
*$P<0.05$; **$P<0.01$.
NBT, nitroblue tetrazolium; TP, total protein in serum.

$P<0.01$), and between NBT reduction activity and total leukocyte count ($r=-0.173$, $P<0.05$) were also found. In the males, a significant correlation between phagocytosis and age was found ($r=0.236$, $P<0.05$). Significant correlations between NBT reduction activity and age ($r=0.218$, $P<0.05$), and between NBT reduction activity and total leukocyte count ($r=-0.308$, $P<0.01$) were also observed. In the females, a significant correlation between phagocytosis and TP was found ($r=0.282$, $P<0.05$), whereas significant correlations between the NBT reduction activity and these factors were not observed.

The relationships between neutrophil functions and stress-coping factors (exercise, and smoking and

TABLE 2. Relationships between the lifestyle factors and the neutrophil functions

Item	Total ($n=147$)[a]		Males ($n=84$)[a]		Females ($n=63$)[a]	
	Phagocytosis	NBT reduction activity	Phagocytosis	NBT reduction activity	Phagocytosis	NBT reduction activity
Age	0.143	0.219**	0.236*	0.218*	0.069	0.077
Subjective stress score	−0.041	−0.078	−0.089	−0.178	0.033	0.016
Total leukocyte count (10^3/mm^3)	−0.069	−0.173*	0.009	−0.308	−0.121	−0.077
TP (g/dl)	0.229**	0.065	0.172	0.071	0.282*	0.204

Item	Total[b]				Males[b]				Females[b]			
	Phagocytosis		NBT reduction activity		Phagocytosis		NBT reduction activity		Phagocytosis		NBT reduction activity	
	n		*n*		*n*		*n*		*n*		*n*	
Stress-coping factor												
≧1	71	8.5±3.7	71	1.1±0.5*	39	8.0±7.8	39	1.2±0.5	32	9.1±3.2	32	1.0±0.6*
0	74	7.8±2.3	74	0.9±0.4	43	7.8±2.3	43	1.0±0.4	31	7.8±2.1	31	0.8±0.4
Exercise habit												
≧1 h/week	106	8.2±3.0	106	1.0±0.5	60	8.0±3.1	60	1.1±0.5	46	8.4±2.8	46	0.9±0.5
<1 h/week	41	8.3±3.2	41	1.0±0.5	24	8.0±3.5	24	1.1±0.5	17	8.7±2.9	17	0.9±0.5
Smoking habit												
Non/quit	117	8.2±2.9	117	1.0±0.5	59	8.0±3.1	59	1.1±0.4	58	8.5±2.8	58	0.9±0.5
Current	30	8.2±3.4	30	1.1±0.6	25	8.1±3.5	25	1.1±0.5	5	8.8±3.1	5	1.1±0.8
Alcohol-drinking habit												
Yes	74	8.4±3.6	74	1.1±0.5	54	8.3±3.7	54	1.1±0.5	20	8.8±3.1	20	1.1±0.8
Non	73	8.0±2.4	73	0.9±0.5	30	7.5±2.0	30	1.1±0.4	43	8.5±2.8	43	0.9±0.5

[a]Values indicate correlation coefficient (r).
[b]Values indicate mean±SD.
*$P<0.05$.
**$P<0.01$.
NBT, nitroblue tetrazolium; TP, total protein in serum.

TABLE 3. Correlations between phagocytosis and NBT reduction activity in various groups

	Total		Males		Females	
	n	*r*	*n*	*r*	*n*	*r*
Stress-coping factor						
≧1	71	0.570**	39	0.673**	32	0.507**
0	74	0.301**	43	0.288	31	0.361*
Exercise habit						
≧1 h/week	106	0.458**	60	0.511**	46	0.455**
>1 h/week	41	0.535**	24	0.564**	17	0.579*
Smoking habit						
Non/quit	30	0.419**	59	0.452**	58	0.451**
Current	117	0.669**	25	0.656**	5	0.812
Alcohol-drinking habit						
Yes	74	0.570**	54	0.596**	20	0.523*
Non	73	0.325**	30	0.249	43	0.461**

Values indicate correlation coefficient (r) between phagocytosis and NBT reduction activity, $^{*}P<0.05$, $^{**}P<0.01$.
NBT, nitroblue tetrazolium; TP, total protein in serum.

alcohol-drinking habits) are shown in Table 2. In all of the subjects, the NBT reduction activity in the group without stress-coping factors was significantly lower than that in the group with one or more stress-coping factors ($P<0.05$). Similar results were also observed in the male and female subjects, and an especially significant effect was observed in the female subjects ($P<0.05$).

Correlations Between Neutrophil Functions in Different Populations

The correlations between phagocytosis and NBT reduction activity in various groups, classified on the basis of lifestyle factors, were examined (Table 3). In all of the subjects, the two correlation coefficients (r) in the groups classified according to stress-coping factors were significantly different ($P<0.05$). Particularly in the males (Fig. 1), there was a significantly positive correlation in the group with one or more stress-coping factors ($r=0.673$, $P<0.01$), while the group without any stress-coping factors showed no significant correlation ($r=0.288$, ns). The correlation coefficients (r) in these groups were significantly different ($P<0.05$). To exclude the age factor, a comparison of the partial regression coefficients (β) was done (dependent parameter: NBT reduction activity; independent parameters: phagocytosis and age), and a significant difference was found (group with stress-coping factor: $\beta=0.642$; group without stress-coping factor: $\beta=0.277$; $z=1.962$, $P<0.05$). A similar tendency was observed in the females,

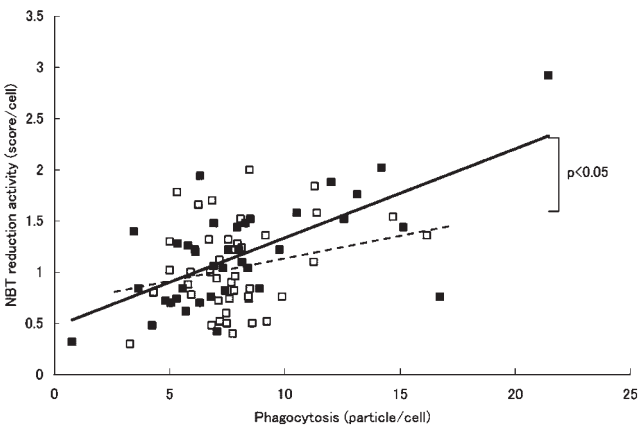

Fig. 1. Correlations between neutrophil functions in elderly males. ■—, group with stress-coping factors ($r=0.673$, $P<0.01$). □—, group without stress-coping factors ($r=0.288$, ns).

although the correlation coefficients (r) in these groups were not significantly different.

In addition, there was a significantly positive correlation in the male group with alcohol-drinking habits ($r=0.596$, $P<0.05$), whereas the group without alcohol-drinking habits showed no significant correlation ($r=0.249$, ns). The correlation coefficients (r) in these groups tended to be different ($P=0.07$; Fig. 2).

Multiple Regression Analysis

The results of multiple regression analysis using neutrophil function as a dependent parameter are shown

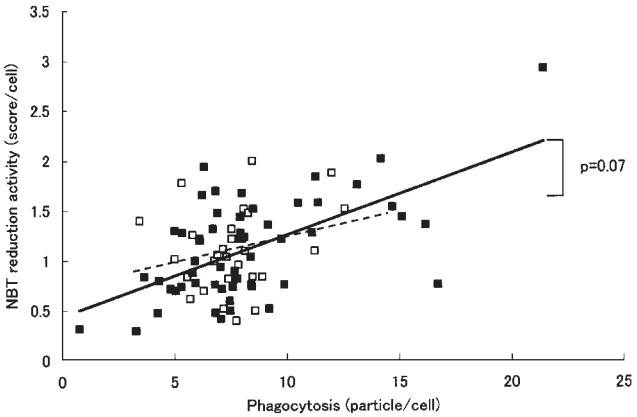

Fig. 2. Correlations between neutrophil functions in elderly males. ■—, group with alcohol-drinking habit ($r = 0.596$, $P < 0.01$). □—, group without alcohol-drinking habit ($r = 0.246$, ns).

in Table 4. Because the superoxide production by the neutrophils was essentially due to phagocytosis, the NBT reduction activity was excluded from the independent parameters when the phagocytosis was used as a dependent parameter. In all of the subjects, age and TP were selected as significant independent parameters of phagocytosis, whereas age, stress-coping factors, total leukocyte count, and phagocytosis were selected as significant independent parameters of NBT reduction activity. In the males, age and alcohol-drinking habit were selected as significant independent parameters of phagocytosis, whereas total leukocyte counts and phagocytosis were selected as significant independent parameters of NBT reduction activity. In the females, TP was selected as a significant independent parameter of phagocytosis, and only phagocytosis was selected as a significant independent parameter of NBT reduction activity.

DISCUSSION

In the present study, a possible gender difference was first examined for the individual observed items (Table 1). The result revealed significant differences not only in the neutrophil parameters but also in the age distribution and serum TP values. The relationships between the lifestyle factors and the neutrophil functions were consequently examined in the male and female subjects separately.

In the males, significantly positive correlations were found between age and phagocytosis, and between age and NBT reduction activity (Table 2). Miyaji et al. (16) reported that phagocytosis of neutrophils in the centenarian group was significantly higher than that in the middle-aged group, whereas superoxide production activity in the centenarian group was significantly lower. A discrepancy thus exists between our results and those of the previous study (16) regarding the relation between aging and superoxide production activity. In the present study, we applied the stimulated NBT reduction test using *Staphylococcus aureus* to evaluate the neutrophil phagocytosis and superoxide production. NBT reduction activity, which represents superoxide production activity, is coupled with phagocytosis of bacteria. In the Miyaji et al.'s (16) study, heat-killed opsonized yeast and human serum were added to the isolated neutrophils to assay phagocytosis. In addition, to determine the superoxide production, isolated neutrophils were stimulated with phorbol mirystate acetate (PMA) and chemiluminescence was used. The aforementioned

TABLE 4. Multipule regression analysis

	Dependent parameter					
	Phagocytosis	NBT reduction activity	Phagocytosis	NBT reduction activity	Phagocytosis	NBT reduction activity
	Total ($n = 145$)		Males ($n = 82$)		Females ($n = 63$)	
Age	0.213*	0.161*	0.395**	0.069	0.101	0.059
Subjective stress score	0.001	0.002	−0.012	0.002	0.003	0.004
Stress-coping factor[a]	0.132	0.151*	0.118	0.153	0.179	0.135
Exercise habit[b]	−0.043	−0.001	−0.057	0.006	−0.033	−0.004
Smoking habit[c]	−0.027	−0.127	−0.101	−0.054	−0.086	−0.101
Alcohol-drinking habit[d]	−0.133	−0.102	−0.261*	−0.021	−0.126	−0.163
Total leukocyte count ($10^3/mm^3$)	0.048	−0.191*	0.042	−0.299*	0.096	0.036
Phagocytosis (particle/cell)	–	0.403**	–	0.496**	–	0.379**
TP (g/dl)	0.291**	0.048	0.216	0.043	0.296*	0.123

[a]Stress-coping factor: having 1 or more = 1,without stress-coping factor = 0.
[b]Exercise habit: 1 h or more/week = 1, below 1 h/week = 0.
[c]Smoking habit: current = 1, non/quit = 0.
[d]Alcohol-drinking habit: non = 1, yes = 0.

discrepancy between the results of superoxide production may be explained by the fact that the process of superoxide production differs depending on the methodology used to detect the oxidant or cell stimulants.

Regarding the relation with the stress-coping factors, the NBT reduction activity in the group without any stress-coping factors was significantly lower than that in the group with one or more stress-coping factors in the total subjects and in the females. However, no difference in phagocytosis was found between the groups (Table 2). Suppression of superoxide production against phagocytosis of bacteria was considered. It has been reported (17) that the presence of stress-coping factors, such as having hobbies or intimate friends, are important for health maintenance. Thomas et al. (18) reported that elderly women who lack social support (close links with families or friends) have significantly lower immune functions than those who have social support. A similar phenomenon was observed in the neutrophil functions examined in the present study.

Concerning the correlation between phagocytosis and NBT reduction activity (Table 3, Fig. 1), a significantly positive correlation was found in the male group with one or more stress-coping factors ($r = 0.673$, $P < 0.01$), whereas no significant correlation was observed in the male group without any stress-coping factors ($r = 0.288$, ns). Moreover, there was a significant difference in the correlation coefficients between these two groups, which was dependent on the age factor ($P < 0.05$). In the females, although no significant difference was found between the two groups in the correlation coefficients, a similar tendency was observed (group with stress-coping factors: $r = 0.507$, $P < 0.01$; group without stress-coping factors: $r = 0.361$, $P < 0.05$). In the total subjects, there was a significant difference in the correlation coefficients between these two groups ($P < 0.05$). We previously reported (14) that the correlations of the neutrophil functions were lost in a stress-loading group, including isolated/overcrowded conditions for 7 days in mice, and in a non-exercise group in rats. This previous study was done on the basis of the hypothesis that stress or lack of exercise diminishes the balance of these neutrophil functions, and we also confirmed these findings in the present epidemiological study for the first time in a human population. The excessive release of superoxides, which are produced as bactericidal agents, was also reported to cause tissue damage (19). This result suggests the importance of examining the balance between phagocytosis and superoxide production. However, no effect of exercise on the balance between neutrophil functions was observed in the present study. Further investigations, including more detailed evaluations of exercise conditions (intensity, duration, and frequency), are needed.

We also found a relationship between alcohol-drinking habits and the balance of neutrophil functions; i.e., there was a significantly positive correlation in the male group with alcohol-drinking habits ($r = 0.596$, $P < 0.05$), whereas the group without alcohol-drinking habits showed no significant correlation ($r = 0.249$, ns). The correlation coefficients in these two groups tended to be different (Fig. 2). In the males, having an alcohol-drinking habit was also found to be a significant independent parameter to the phagocytosis (Table 4). It is known that moderate alcohol drinking can be good for health (20). Since alcohol intake by the subjects in the present study was considered almost moderate, it may have had a beneficial effect on the balance of the neutrophil functions.

A relationship between smoking and neutrophil functions was not indicated by the results of the present study; however, Ludwig et al. (21) reported that smokers with elevated peripheral leukocyte counts ($> 9{,}000/mm^3$) have an increased release of superoxide by neutrophils. In the present study, tobacco consumption by the current smokers was considered to be comparatively light, and almost all current smokers among our subjects showed leukocyte counts of less than 9,000. It was concluded that smoking habits in the present subjects did not remarkably affect the leukocyte counts and neutrophil functions.

As regards the relationships between the serum TP level and the neutrophil functions (Table 2), a significant correlation between phagocytosis and TP was found in the total subjects ($r = 0.229$, $P < 0.01$) and the females ($r = 0.282$, $P < 0.05$). Furthermore, the results of multiple regression analysis (Table 4) showed a similar tendency. The decrease in neutrophil function due to protein deficiency is well known (22), and some authors further investigated its relevancy to aging (11,23). Lipschitz et al. (11) showed that poor protein intake resulted in markedly decreased the phagocytic and killing capabilities of the neutrophils in rats, and they emphasized that the neutrophil functions were markedly disturbed when other factors, such as poor nutrition, were added to the age factor. However, the low serum TP level observed in the subjects was not so remarkable as to be called a "protein deficiency." Since the NBT reduction test is a technique performed without isolating the neutrophils from the whole blood, opsonin factors (including complement and immunoglobulin) affect the phagocytosis of the neutrophils. The relation between the TP level and phagocytosis may be attributable to the low opsonin activity that was represented by the serum TP level, which resulted in reduced phagocytosis. Adequate protein intake is apparently important for maintaining neutrophil functions in the elderly.

The results of the present study indicated that there is a relationship between neutrophil functions and lifestyle factors in the elderly. In particular, we suggest that aging, TP, and stress-coping factors affect the neutrophil functions. In addition, significant effects of lifestyle factors on the balance between phagocytosis and subsequent superoxide production were found. The results obtained from the males did not conform completely with those from the females. The differences in mean age, lifestyles, and serum protein levels between the males and females (Table 1) may explain this discrepancy.

Treating stress and attaining adequate serum TP levels appears to be very important for preventing infectious diseases in the elderly. The elderly subjects examined in the present study received guidance in treating stress and maintaining an adequate nutritional state. Consequently, the levels of subjective stress, hypertension, and serum TP were clearly improved (24). It is therefore necessary to examine these parameters, including their effects on neutrophil functions, in more detail in the future.

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