Original Article

Relationship of aging and nutritional status to innate immunity in tube-fed bedridden patients

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Background and Objectives: Aging and malnutrition are known to influence immune functions. The aim of this study was to investigate the relationship of aging and malnutrition to innate immune functions in tube-fed bedridden patients. **Methods and Study Design:** A cross-sectional survey was performed in 71 tube-fed bedridden patients aged 50-95 years (mean age±SD, 80.2±8.5 years) with serum albumin concentrations between 2.5 and 3.5 g/dL. We evaluated associations of age and nutritional variables with natural-killer cell activity, neutrophil-phagocytic activity, and neutrophil-sterilizing activity. Nutritional variables included body mass index, weight-adjusted energy intake, total lymphocyte count, and serum concentrations of albumin, transferrin, prealbumin, total cholesterol, C-reactive protein, and zinc. **Results**: Natural-killer cell activity, neutrophil-phagocytic activity, and neutrophil-sterilizing activity were normal or increased in 67 (94%), 63 (89%), and 69 (97%) patients, respectively. Multiple linear regression analysis with a backward elimination method showed that natural-killer cell activity correlated negatively with aging and lymphocyte counts ($p<0.01$ for both) but positively with body mass index and transferrin ($p<0.01$ for both). Neutrophil-phagocytic and neutrophil-sterilizing activities were not associated with any variables. **Conclusions**: In tube-fed bedridden patients with hypo-albuminemia, natural-killer cell activity may be associated with aging, body mass index, transferrin, and lymphocyte counts.

Key Words: aging, natural-killer cell activity, neutrophil function, nutritional status, tube feeding

INTRODUCTION

Although the human body is continuously exposed to pathogenic microbes, orchestration of the immune system consisting of innate and adaptive immunity successfully protects against infection. Innate immunity provides the first-line of defense. Natural killer (NK) cells, neutrophils and antigen-presenting cells, the main effector cells of innate immunity, immediately respond upon encountering pathogenic microorganisms. The innate immune response is followed by adaptive immunity, which is pathogen-specific and more stringent. Thus, innate immunity plays a crucial role in the early stage of immune responses to offending pathogens.

As the development of critical care expands for elderly population, the number of tube-fed bedridden patients in Japan is expected to increase.[1] Tube-fed bedridden patients tend to be malnourished despite adequate nutritional support.[2,3] Malnutrition appears to compromise immune functions, leading to increased susceptibility to infection.[4,5] In addition, immune functions are impaired in parallel with aging.[6] Therefore, elderly tube-fed bedrid-

den patients have a higher risk for infectious diseases and thus, an understanding of immune functions in elderly patients may be helpful for infection control.

The purpose of the present study was to examine the innate immunity of elderly tube-fed bedridden patients. We particularly focused on NK cell activity, neutrophil-phagocytic activity, and neutrophil-sterilizing activity because these parameters have been used as objective indices of innate immune functions. We also examined whether aging and nutritional status were associated with NK cell activity, neutrophil-phagocytic activity, and neutrophil-sterilizing activity.

METHODS

Study design

The study design was cross-sectional. This was a secondary analysis of data from a multicenter randomized trial where tube-fed patients randomly received enteral formula including lactoferrin or control formula for 12 weeks.[7] The daily calories received were not changed in the previous 4 weeks to avoid influences from a change in calories given. After overnight fasting, blood samples were collected for determination of immunological and biochemical nutritional variables.

Patients

The study patients were recruited between September 2008 and December 2009 at Sangenjaya Dai-ichi Hospital, Sangenjaya Hospital, Fuji-Yoshida Municipal Hospital, Ohta-Atami Hospital, and Kohga Hospital in Japan. They consisted of 71 consecutive tube-fed bedridden patients (40 men and 31 women) aged 50-95 years (mean age±SD; 80.2±8.5 years) with serum albumin concentrations between 2.5 and 3.5 g/dL. All patients had co-morbid conditions, which resulted in swallowing difficulties. Almost all co-morbid conditions were due to cerebrovascular diseases. The patients were completely dependent upon artificial nutrition and hydration through percutaneous endoscopic gastrostomy (n=58), nasogastric tubing (n=11), or percutaneous trans-esophageal gastro-tubing (n=2) with a mean duration of 19.7 months (range; 1-82). Patients with any of the following criteria were excluded: food allergies, liver failure, severe renal dysfunction, or diagnosed as having cancer.

Immunological variables

Immunological variables included NK cell activity, neutrophil-phagocytic activity, and neutrophil-sterilizing activity.[8-11] The determination of immunological variables obtained from blood samples was performed at SRL (Hachioji, Japan).

NK cell activity was determined by ^{51}Cr-release assay as follows: total lymphocytes were isolated using Ficoll-Conray solution, followed by incubation with K-562 cells labeled with ^{51}Cr. Thereafter, released soluble ^{51}Cr was measured by gamma-counter.

Phagocytic and sterilizing activities of neutrophils were determined using the method described by Steinkamp et al.[12] Briefly, for phagocytic activity measurements, 100 µL of heparinized whole blood was placed in a polypropylene tube and 5 µL of fluorescent carboxyl particle (Fluoresbrite® YG Carboxylate Microspheres 2.00 µm, Polysciences, Inc) was added. Then, cells were incubated for 45 mins at 37°C. After incubation, cells were washed with PBS containing 3 mM EDTA and erythrocyte lysing solution was added. Then, cells were washed with PBS containing 3 mM EDTA and resuspended in PBS. The phagocytic activity of these cells was measured by FACScan flow cytometer.

For measurement of sterilizing activity, 100 µL of heparinized whole blood was placed in a polypropylene tube and incubated with 5 µM of DCFH (2', 7'-dichlorodihydrofluorescin) solution for 15 mins at 37°C. After incubation, cells were washed with 25 mM EDTA in PBS and stimulated with 50 µg/mL PMA (phorbol myristate acetate) for 25 mins at 37°C, and then washed with 25 mM EDTA in PBS. Erythrocyte lysing solution was added and cells were suspended in 2 mL of 25 mM EDTA in PBS. The sterilizing activity was measured by FACScan flow cytometer.

The reference range of each variable was as follows: NK cell activity (18-40%), neutrophil-phagocytic activity (80-100%), and neutrophil-sterilizing activity (80-100%).

Nutritional variables

Nutritional variables included total lymphocyte counts (TLC) and serum concentrations of albumin, transferrin, prealbumin, total cholesterol (TC), and C-reactive protein (CRP) according to Omran et al.[13] In addition, serum zinc concentrations were examined since it was reported to be essential for immune functions.[14] The BMI and weight-adjusted energy intake were also included as nutritional variables. The determination of nutritional variables obtained from blood samples were performed at SRL (Hachioji, Japan).

Albumin concentrations were determined using the bromocresol green method. Transferrin and prealbumin concentrations were determined using the turbidimetric immunoassay. TC concentrations were determined using the enzymatic method. CRP concentrations were determined using nephelometry. TLC was computed as the white blood cell multiplied by the total percentage of lymphocytes, using an automated blood cell counter. Zinc concentrations were determined using atomic absorption spectrometry. To determine BMI, the patients' height was estimated by measuring their full length on the bed and BMI was calculated by dividing body weight with their height squared.

The reference range of each variable was as follows: albumin (4.0-5.0 g/dL), transferrin (190-300 mg/dL), prealbumin (22-40 mg/dL), TC (150-219 mg/dL), CRP (≤0.3 mg/dL), TLC (1,500-4,000 cells/µL), and zinc (65-110 µg/dL). A BMI of 18.5 kg/m^2 is recognized as the lower limit for healthy weight.[15]

Ethical considerations

Each hospital initiated the study after approval by the appropriate ethical committee. Written informed consent was obtained from the next-of-kin after a full explanation, as patients could not communicate appropriately.

Statistical analysis

The data were presented as the mean±SD. Pearson's correlation coefficients were used to examine the linear relationship of age and each nutritional variable to immunological variables. Multiple regression analysis with backward elimination method (elimination criterion, $p>0.15$) was used to identify significant factors independently contributing to the immunological variables. Levels of significance were set at 0.05 (two-sided) for all statistical analyses. All calculations were conducted using SAS version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

The demographics of the participants are shown in Table 1. Forty-seven participants (66%) presented with a BMI of less than 18.5 kg/m^2. In addition, blood concentrations

Table 1. Demographic and clinical characteristics of the patients (n=71)

Variable	Overall	Type of tube feeding		
		Gastrostomy (58)	Nasogastric (11)	Esophagostomy (2)
Age (years)	80.2±8.5	80.2±8.8	81.2±5.8	76.5±14.8
Sex: men/women (n)	40/31	33/25	7/4	0/2
Height (cm)	155±9.6	156±19.7	151±9.4	147±5.3
Body weight (kg)	42.5±7.9	42.7±9.0	41.5±5.0	40.9±11.0
Body mass index (kg/m^2)	17.8±3.3	17.6±3.7	18.4±2.9	18.8±3.7
Midarm circumference (cm)	21.0±3.3	21.3±5.1	20.9±1.5	21.1±4.0
Calf circumference (cm)	25.1±6.1	25.3±6.7	24.8±5.4	25.0±4.3
Triceps skinfold thickness (mm)	11.1±5.7	11.8±5.6	10.4±5.8	10.6±5.6
Energy intake (kcal/day): overall	1017±188	1038±215	927±119	900±141
Weigh-adjusted energy intake (kcal/kg/day): overall	24.5±5.2	24.9±5.7	22.5±2.8	23.3±9.7
Protein intake (g/day)	46.7±8.9	47.4±10.1	44.9±4.2	36.0±5.7
Weight-adjusted protein intake (g/kg/day)	1.1±0.3	1.1±0.3	1.1±0.1	0.9±0.4
Duration of tube-feeding dependence (months)	19.7±21.6	19.4±22.3	21.3±20.3	21.0±17.0
Natural killer cell activity (%)	38.5±14.9	37.8±14.9	40.1±15.8	51.0±12.7
Neutrophil-phagocytic activity (%)	90.9±8.8	90.7±8.9	91.3±8.7	96.7±2.7
Neutrophil-sterilizing activity (%)	96.2±5.3	96.8±4.6	92.5±7.5	99.3±0.8
Albumin (g/dL)	3.1±0.2	3.1±0.2	3.2±0.3	3.4±0.0
Transferrin (mg/dL)	192±43.7	189±43.7	201±42.4	224±60.1
Prealbumin (mg/dL)	17.9±5.1	17.8±5.1	18.0±6.0	18.1±1.1
Total cholesterol (mg/dL)	158±33.0	156±32.4	167±38.4	168±20.5
C-reactive protein (log mg/dL)[†]	0.6±4.5	0.6±4.8	1.0±2.6	0.1±5.1
Zinc (µg/dL)	54.0±11.2	53.9±11.8	54.4±8.9	56.0±1.4
Lymphocyte counts (per µL)	1839±679	1871±727	1691±358	1707±762
Neutrophil (per µL)	4239±2066	4344±2207	3822±1116	3479±2228

Values represents the mean±SD.
[†]C-reactive protein concentrations are log-transformed for analysis.

Table 2. Correlation of study variables with natural killer, neutrophil-phagocytic, and neutrophil- sterilizing activities

	Natural killer activity	Neutrophil-phagocytic activity	Neutrophil-sterilizing activity
Age	-0.297[**]	-0.156	-0.138
Body mass index	0.288[*]	-0.069	-0.169
Albumin	0.111	0.058	-0.024
Transferrin	0.192	0.187	-0.094
Prealbumin	0.018	-0.185	-0.027
Total cholesterol	0.072	-0.211	-0.066
C-reactive protein[†]	-0.090	0.095	0.007
Zinc	-0.019	0.047	-0.053
Lymphocyte counts	-0.160	-0.046	-0.135
Weigh-adjusted energy intake	-0.102	0.060	0.158

Data are presented as the correlation coefficients.
[†]C-reactive protein concentrations are log-transformed for analysis.
[*]$p < 0.05$, [**]$p < 0.01$.

of transferrin, prealbumin, TC, zinc, and TLC were lower than the reference range in 36 (51%), 59 (83%), 31 (44%), 56 (79%), and 26 (37%) patients, respectively. Fifty-two patients (73%) presented with higher CRP concentrations than the reference range. Surprisingly, the mean values of NK cell activity, neutrophil-phagocytic activity, and neutrophil-sterilizing activity were within the reference range. Only 4 patients (6%), who were aged >80 years and with a BMI <18.5 kg/m^2, presented with NK cell activity lower than the reference range. Neutrophil-phagocytic and neutrophil-sterilizing activities were lower than the reference range in 8 (11%) and 2 (3%) patients, respectively.

The results of Pearson's correlation coefficients are shown in Table 2. The analysis showed that NK cell activity was negatively correlated with age ($p < 0.01$) and positively correlated with BMI ($p < 0.05$). All other variables including serum albumin concentrations were not

associated with NK cell activity. Both neutrophil-phagocytic and neutrophil-sterilizing activities were not associated with any of the variables examined.

As shown in Table 3, multiple regression analysis revealed that NK cell activities were positively associated with BMI and transferrin ($p < 0.01$ for both) and were negatively associated with age and TLC ($p < 0.01$ for both) after adjusting for other variables. No specific variables were found to be associated with the phagocytic and sterilizing activities of neutrophils.

DISCUSSION
Tube-fed bedridden patients present a difficulty for health maintenance, as nutritional support is completely dependent upon artificial nutrition and hydration, and physical activities are extremely impaired. Therefore, it is likely that their immune functions are different from those of

Table 3. The final regression coefficients of multiple-regression analyses with backward elimination method for natural killer cell activities on independent variables

Independent variables[†]	Regression coefficient (β)
Age	-0.578±0.184[**]
Body mass index	1.45±0.476[**]
Transferrin	0.119±0.038[**]
Lymphocyte counts	-0.007±0.002[**]

Data are presented as the regression coefficient (β)±SE.
[†]Variables at onset: age, body-mass index, albumin, transferrin, prealbumin, total cholesterol, C-reactive protein, zinc, lymphocyte counts, and weight-adjusted energy intake.
[*]$p<0.05$, [**]$p<0.01$.

healthy subjects. This study is the first to examine immune cell functions of Japanese tube-fed bedridden patients. Our results showed that NK cell activity, neutrophil phagocytosis, and neutrophil-sterilizing activity mostly remained within the normal range. However, we observed that NK cell activity was significantly associated with aging, BMI, transferrin, and TLC.

As patients in this study were elderly (mean age; 80 years) and malnourished (albumin concentrations; 2.5-3.5 g/dL and mean BMI; 17.8 kg/m^2), we were surprised to observe that NK cell activity and neutrophil functions were mostly within the normal reference range. A simple interpretation of the results is that innate immune functions are maintained even in tube-fed bedridden patients. However, it should be noted that serum CRP concentrations were substantially increased. Although the reason for this is unknown, the data indicated that the patients were suffering from disease conditions, which likely triggered inflammatory responses at the time of participation. As the activity of NK cells and neutrophils are partly regulated by pro-inflammatory cytokines,[16] it is likely that normal levels of NK cell activity and neutrophil functions we observed reflected such inflammatory responses. Alternatively, if the calorie intake was not sufficient, variable immunomodulatory effects due to calorie restriction could have affected normalization of NK cell activity and neutrophil functions.[17,18]

Previous studies examining the influence of aging on NK cell functions, found inconclusive results in healthy elderly subjects.[8,9,19] In this study population, NK cell activity was negatively correlated with aging. Our results appear to be consistent with a report by De la Rosa et al who showed that overall NK cell cytotoxicity was diminished in non-healthy and frail elderly patients.[20] Therefore, NK cell activity may decrease due to the aging process in tube-fed bedridden patients. However, subjects in our study had co-morbid conditions. Thus, we cannot exclude the possibility that such co-morbid conditions influenced the effect of aging on NK cell activity by affecting immune functions.[21]

BMI was found to be positively associated with NK cell activity, indicating that poor nutritional status may lead to a decrease in NK cell activity. Indeed, four patients who presented with NK cell activity lower than the normal reference range had BMI <18.5 kg/m^2. The result is consistent with that of Villa et al and Salimonu et al who reported that NK cell activity was associated with nutritional status in cancer patients and children.[22,23] Transferrin concentrations were also positively associated with NK cell activity. Since transferrin is a primary carrier protein of iron and is required for NK cells to develop cytotoxic activity,[24] our results indicated that the amount of serum transferrin present may influence NK cell activity. Among other nutritional variables, TLC was negatively associated with NK cell activity although the regression coefficient is substantially low. The reason for this is uncertain. Similar to the relationship of NK cell activity in the elderly,[25] an increase in TLC might be compensatory in nature as a response to a decrease in lymphocyte functions, such as cytokine release or proliferation, which can affect NK cell activity. However, the level of albumin, commonly used to measure nutritional variability, showed no association in regression analyses. A possible reason for the inconsistency is that the subjects of this study were pre-specified: the serum albumin concentration was restricted to being between 2.5 to 3.5 g/dL.

Unlike NK cell activity, neutrophil-phagocytic and neutrophil-sterilizing activities were not associated with aging, BMI, transferrin, or TLC in multiple regression analyses. Other nutritional variables of interest did also not correlate with neutrophil functions. Although several studies have attempted to investigate the effect of aging and nutrition on neutrophil functions, the results have been inconsistent. In some studies, decreased neutrophil-phagocytic and neutrophil-sterilizing functions in elderly or malnourished patients, were attributable to an impaired reactivity to cytokines or a decrease in intracellular metabolic systems necessary for neutrophil functions.[10,26-30] In contrast, other studies did not support this notion.[11,31,32] Such a discrepancy may be due to the study population and/or methodological differences between the studies. Likewise, the patients' co-morbidities, disease history, and medication used, all of which can modulate immune functions, might have influenced the neutrophil functions in this study.[19] Further studies are needed to conclude this issue.

There are a number of limitations in this study. First, the sample size (n=71) is small which may result in low statistical power. Second, nutritional support at the time of participation was not standardized. The amount of calories and proteins administered were not strictly weight-adjusted, so variations in the basal nutritional status of patients may have confounded the observed results. Third, because detailed clinical work-ups of the patients were mostly impossible, presence of preclinical malignancies and/or subclinical infectious diseases which are potent confounders were not completely excluded. Fourth, medications at the time of recruitment was not incorporated for the analysis. Lastly, the study population did not fulfill the strict criteria suggested by the SENIEUR protocols, indicating that interpretation of the results must be cautious.[19] Nonetheless, our data provide a new insight into understanding the innate immune functions in elderly tube-fed bedridden patients.

In conclusion, the current study showed that almost all of the tube-fed bedridden patients had an equal to or higher level of NK cell activity, neutrophil-phagocytic activity, and neutrophil-sterilizing activity compared with normal healthy subjects. NK cell activity was associated

with aging, BMI, transferrin, and TLC while phagocytic or sterilizing activities of neutrophils were not associated with aging and nutritional variables.

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