福大医紀（*Med. Bull. Fukuoka Univ.*）：30(1), 29–35, 2003

Age-related Dissociation of Oxidative Metabolisms from Phagocytosis and Up-regulation of CD11b/CD18 Expression in Human Monocytes

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Abstract : Immunosenescence is one of the crucial events in aging, resulting in a vulnerability to infection and an increased incidence of malignant tumors. In the present study, we investigated the activities of phagocytic cells, including polymorphonuclear neutrophils (PMN) and monocytes, from elderly persons, and compared these findings to those in young subjects. The production of reactive oxygen intermediates (ROI) in the peripheral whole blood by stimulation with fresh serum-opsonized zymosan was significantly augmented in elderly persons in comparison to that in young volunteers, when estimated by a luminol-dependent chemiluminescence (CL) assay. The CL response of PMN showed a similar level in both groups, but that of monocytes increased remarkably in the elderly group. On the other hand, phagocytosis of fluorescent microspheres by monocytes, observed by flow cytometry, significantly decreased in the elderly, whereas the expression of CD11b/CD18 (CR3) which is related to the phagocytic production of ROI and phagocytosis *per se* was up-regulated in monocytes from elderly persons, in comparison to the younger subjects. These results suggest that functional changes in the phagocytes due to aging occurred more deeply in monocytes than PMN, which might therefore represent one of the characteristics occurring in immunosenescence.

Key words : Monocytes, Reactive oxygen intermediates (ROI), Phagocytosis, CD11b/CD18, Aging

Introduction

Immunosenescence in aging is associated with an increased occurrence of infection, malignant tumor, and certain types of autoimmune disease.[1)-3)] There have been many reports, in this regard, examining the age-related changes in some parameters of the host immune system, mainly on lymphocyte-mediated humoral and cellular immunity (reviewed in 4). It is generally agreed that T cell responses in the elderly decrease in comparison to those in young individuals, though some reports are in conflict on minor points ; e.g., some report an increase in the CD4/CD8 ratio or IFN-γ production in human peripheral blood lymphocytes with age,[5)6)] while the others show a contrary trend.[7)8)] As for phagocytic cells (phagocytes) representing non-specific immunity, their functions seem to decline with age on the whole,[9)-11)] but there are, still, many studies yielding controversial results ; neither signifi-

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cant differences between elderly and young, nor significant decreases in the elderly have been reported.[12)–14)] This variance may be due to the features of the subjects examined. In fact, the immunocompetent cells may be influenced by many factors, such as, diet, exercise, illness, drug treatment or psychiatric stresses.[15)–17)]

Phagocytes including polymorphonuclear neutrophils (PMN) and monocytes/macrophages play a critical role in the clearance of invading microbes through their phagocytic capacities. They 'recognize' these microbes mainly via plasma membrane receptors for complement fragments (CR or C3R) or the Fc region of IgG (FcγR).[18)19)] Upon coming in contact with those targets, they also generate reactive oxygen intermediates (ROI) via the activation of NADPH-dependent oxidase,[20)] following a sharp increase in oxygen consumption known as 'respiratory burst'.[21)] ROI such as those categorized in the free radicals, play a role in the killing of pathogens[21)] or, even of cancer cells,[22)] but they can also be toxic to normal cells or tissues, thus resulting in the occurrence of refractory inflammations or malignant tumors (reviewed in 23). In this study, we examined the functional activities of phagocytes from 'healthy' elderly persons (65–85 years old), and compared the findings to those of young volunteers (20–45 years old). The results indicate that both ROI production in response to serum-opsonized zymosan of monocytes and the expression of relevant receptors, CR3 (CD11b/CD18), on their cell membranes were significantly enhanced in the elderly subjects, whereas their phagocytic activity was impaired. This discrepancy may be associated with the presence of immunosenescence in the elderly.

Materials and Methods

Subjects : Thirty five subjects aged between 65 and 85 years old, of both sexes (23 women and 12 men) were selected from among elderly volunteers. Some of them had mild hypertension, but none had debilitating diseases such as diabetes mellitus, renal insufficiency or malignant tumors. Healthy young volunteers aged 20 to 45 yrs (14 women and 11 men) were chosen to serve as controls. Most of the participants in this study were non-smokers, except for some who smoked only a few cigarettes a day. Heparinized peripheral blood samples were obtained from them to either evaluate their leucocyte counts or to separate their blood cells for ongoing experiments. All of the participants in this study gave their informed consent, after having been informed carefully of the purpose and protocol. The experiment complied with the current laws of Japan.

Separation of polymorphonuclear neutrophils (PMN) and mononuclear leukocytes : PMN were separated by using Ficoll-Paque density gradient centrifugation, followed by dextran sedimentation as described previously.[24)] Monocytes were separated as follows. The mononuclear cell layer obtained by Ficoll-Paque gradient (Pharmacia, Uppsala, Swed.) was washed twice with phosphate-buffered saline (PBS, pH 7.4), re-suspended in phenol red-free Eagle's Minimum Essential Medium (Nissui Seiyaku, Tokyo, Japan) containing 5 mM HEPES buffer (H-MEM, pH 7.4), and then incubated in plastic Petri dishes (FALCON, Oxnard, CA, USA) for 1 hr at 37℃ in a humidified atmosphere of 5% CO_2 in air. After removing any non-adherent cells, adherent cells were collected by vigorous pipetting on ice, and then were regarded as monocytes after a microscopical examination. The viability of the cells, as measured by trypan blue exclusion, was greater than 90%.

Luminol-dependent chemiluminescence (CL) assay : A CL assay was used to evaluate the respiratory burst of phagocytic cells, according to the method of Faden and Maciejewski[25)] with a minor modification of Kuroiwa et al. .[24)] Briefly, a heparinized peripheral blood sample including 5×10^5 leukocytes, or PMN or monocyte suspension (containing 1×10^5 cells, each) was resuspended in 500 μl H-MEM in each test vial and then was incubated for 10 min at 37℃ with the addition of luminol (20 μl of 2 mg/ml solution), and thereafter was stimulated with 20 μl of fresh serum-opsonized zymosan (Sigma, St. Louis, MO, USA : 2

mg/ml). CL was measured at 37℃ in a Biolumat LB9505 (Berthold, Wildbad, Germany).

Phagocytosis assay : Phagocytosis was assessed by estimating the uptake of fluorescent microbeads. A hundred μl of fluorescent microsphere (Fluoresbrite ; Funakoshi, Tokyo, Japan) suspension containing 4×10^7 particles/ml was added to a 100 μl of heparinized peripheral blood. After incubation at 37℃ for 20 min, red blood cell were lysed with FACS Lysing Solution (Becton Dickinson, San Jose, CA, USA). The uptake of the beads by PMN and monocytes was analyzed flow cytometrically using a FACScan (Becton Dickinson).

Surface marker analysis : The expression of CD11b/CD18 (CR3, Mac-1) on monocytes was quantified by direct immunofluorescent staining using FITC-conjugated mAb anti-Leu-15 (Becton Dickinson). A flow cytometric analysis of fluorescence positive cells was performed with a FACScan (Becton Dickinson).

Statistical Analysis : Results are presented as the mean ± SE, unless otherwise indicated. Comparisons of the groups were performed by Student's paired *t*-test. Statistical significance was denoted by $P<0.05$, $P<0.01$ or $P<0.001$, and $P<0.05$ was considered significant.

Results

Peripheral White Blood Cell Counts

An analysis of the influence of aging on peripheral white blood cell counts showed no significant differences ($p>0.05$) in the total leukocyte number or in the number of neutrophils, monocytes and lymphocytes in the elderly people examined, as compared with healthy young volunteers (Table 1).

CL Response of Whole Blood, PMN and Monocytes

Fig. 1 shows CL responses, upon stimulation with fresh serum-opsonized zymosan, in whole blood (A), PMN (B) and monocytes (C). The values in elderly subjects of whole blood and monocytes significantly increased, though they varied widely ranging from control levels to nearly 10-fold values in comparison to those of younger subjects whose results were lower and much less varied. The response of PMN showed no significant difference between the two groups.

Phagocytic Activity of PMN and Monocytes

The uptake of fluorescent microbeads by PMN and monocytes in whole blood was assessed by flow cytometry. Table 2 shows the percentages of fluorescence positive cells or cells phagocytosing microbeads. The activity of the elderly monocytes was significantly reduced, in comparison to that of the younger subjects. There was no significant difference in the activity of PMN between the two groups ($p>0.05$), but a downward trend was observed in the elderly PMN.

Expression of CD11b/CD18 (CR3, Mac-1)

The production of ROI upon fresh serum-opsonized zymosan stimulation and the uptake of microbeads in the presence of serum are supposed to occur mainly through the

Table 1. Peripheral white blood cell counts in young and elderly subjects

	Young	Elderly
Total white cells	6,163±1,375	5,976±1,540
Neutrophils (PMN)	3,286±830	3,341±1,096
Monocytes	407±125	328±150
Lymphocytes	2,318±675	2,107±786
Others	147±52	201±64

Data are expressed as the mean values±SD.
Differences between both groups were not significant ($p>0.05$).

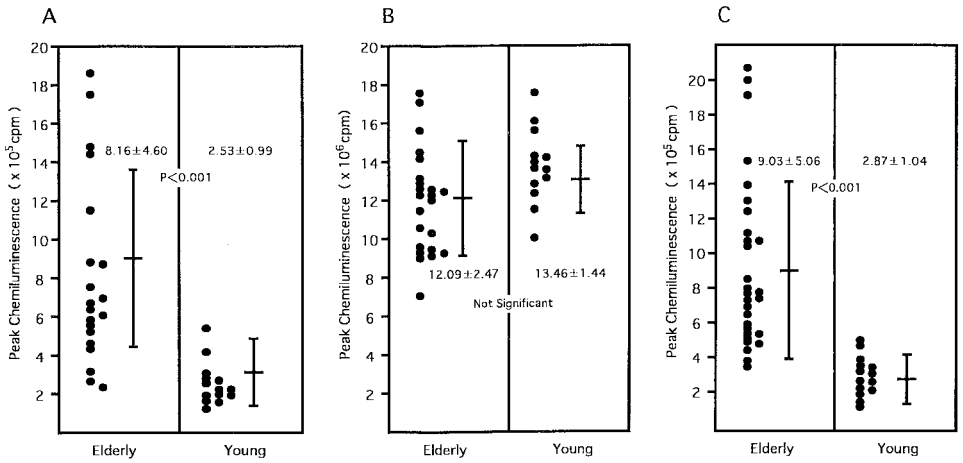

Fig. 1. Chemiluminescence (CL) responses representing ROI production upon stimulation with opsonized zymosan in whole blood (A), PMN (B), and monocytes (C). The mean value (±SD) is denoted in each group.

Table 2. Uptakes of fluorescent microbeads by PMN and monocytes from young and elderly subjects

	Young	Elderly
PMN	67.1±9.7	60.8±7.4
Monocytes	61.4±11.2	48.3±10.6*

Data represent the percentages of fluorescent positve cells (±SD). *$p<0.01$

binding of those particles with a relevant surface receptor of phagocytic cells, *i.e.*, CR3 (or CD11b/CD18), and therefore we investigated the expression of CR3 on PMN and monocytes. As shown in Table 3, the receptor expression was significantly up-regulated on the surface of monocytes from the elderly subjects. Its expression on PMN was not significantly different between the two groups, but the mean fluorescent intensity was slightly stronger on the cells from elderly subjects.

Discussion

PMN and monocytes/macrophages known as 'phagocytes' are considered to exert their function on the first line of the host defense system. Their functional activities are characterized by the following capacities: chemotaxis, phagocytosis, degranulation, nitroblue tetrazolium (NBT) reduction, killing of microbes or tumor cells, production and release of certain cytokines such as IL-1β and TNFα, and production of ROI and RNI (reactive nitrogen intermediates). Many studies have been made to identify any age-related alterations in these activities, but it still remains inconclusive as to how aging affects the phagocytic functions. Generally, however, some functions seem to be declining with age; some authors have indicated a significant decline in chemotactic and phagocytic activities of PMN and/or monocytes,[9)11)26)] as well as in the LPS-induced tumoricidal activity and IL-1 production.[10)] ROI production or oxidative respiratory burst in PMN seems unchanged or rather augmented, when stimulated with opsonized zymosan particles, chemotactic peptide fMeth-Leu-Phe (fMLP), or phorbol myristate acetate (PMA).[9)11)27)]

In the present study, we observed a mark-

Table 3. Peripheral blood CR3 (CD11b/CD18) positive monocytes

	Young	Elderly
Percent positive cells	64.8±10.3	88.9±5.5*
Mean fluorescent intensity	156.7±31.2	163.0±35.6

*$p < 0.001$

edly enhanced luminol-dependent CL response representing the ROI production in peripheral whole blood and in monocytes from elderly persons, upon stimulation with fresh serum-opsonized zymosan (Fig. 1: A, C). The CL in PMN, however, showed no significant difference between the young and elderly subjects (Fig. 1: B), which also correlates with the results of other groups.[9)27)] Since the CL response in whole blood is thought to depend mainly on the response of PMN,[24)] the discrepancy of our results seems to account for the presence, notably in the serum, of factors regulating ROI, or, for the presence of antioxidants or scavengers such as superoxide dismutase (SOD), vitamins C and E, carotenoids, plasma proteins (transferrin, ceruloplasmin, etc.), and others.[28)] Some recent studies in humans demonstrate the existence of a concomitant decrease in most of those antioxidants in the blood of the elderly,[29)30)] which may lead to an increase in the whole blood CL response in the elderly. Furthermore, erythrocyte antioxidant enzymes such as SOD, catalase (CAT) and glutathione peroxidase (GPX), may also affect the response of CL in whole blood assay system, because their enzymatic activities are also thought to decrease in the elderly.[28)]

On the other hand, the CL response of monocytes *per se* increased significantly more in the elderly subjects, than in the young controls (Fig. 1C). As previously described, phagocytic cells generate ROI on the contact with targets via plasma membrane receptors like CR or FcR,[18)19)] following a sharp increase in oxygen consumption.[21)] It is thus likely that the enhanced response of CL in monocytes is closely correlated to the up-regulation of CR3 (CD11b/CD18) expression on the surface membrane of monocytes (Table 3). The exact reasons for this finding have yet to be elucidated. However, it may be possible that, in the elderly subjects, the consistent and persistent contact with inflammatory stimuli, such as microbes in their relatively longer life plays some role in the up-regulation of receptors on the plasma membrane of phagocytes.[31)] In fact, some reports have indicated that lipopolysaccharide (LPS), a product of Gram-negative bacterial cell walls, and proinflammatory cytokines like TNF-α, IL-18, and the macrophage inflammatory protein 1 (MIP-1), provoke the up-regulation of CD11b/CD18 expression.[32)-34)] Esparza et al.[11)] and Leung et al.[34)] also reported an age-related enhancement of CD11b expression on neutrophils, but we did not make such an observation in our findings.

Another feature of our results is a significant decline in phagocytic activity in the elderly monocytes in comparison to the younger ones, although this phenomenon was not seen in PMN (Table 2). Since our study on phagocytosis was performed using fluorescent-conjugated microbeads in the presence of serum, their uptake by phagocytes might be mediated through complement receptors, namely, CR1 and CR3. As shown above, the expression of CR3 (CD11b/CD18) significantly increased in the elderly monocytes, thus resulting in their enhanced production of ROI. How and why does this discrepancy occur? Possibly, the difference in the intracellular signal transduction pathway between ROI production and phagocytosis could be involved in this phenomenon. Phagocytes, upon contact with targets, generate ROI via activation of NADPH-dependent oxidase,[20)] known as respiratory burst, and ROI themselves are thought to function as second messengers in the phase of signal transduction.[35)] In addition, Forsberg et al. recently reported that the p38 MAPK (mitogen-activated protein kinase) was involved in the CR3-induced respiratory burst.[36)] On the other hand, CR3

–mediated phagocytosis, as well as FcR–mediated one, has been reported to play a role in the activations of PLD (phospholipase D) and PTKs (protein tyrosine kinases) belonging to Src family.[37)38)] Since the differences in respiratory burst and phagocytosis of the elderly monocytes, observed in this study, may be due to an alteration(s) in the signal transduction pathway, further studies are needed to precisely elucidate the cause(s) of its alteration, especially in relation to immunosenescence.

Immunosenescence occurs in various types of immunocompetent cells including phagocytic cells and lymphocyte populations,[4)] leading to an impairment or alteration of some of their functions. In particular, a malfunction of phagocytes results in severe infections in the elderly persons, because they exert their function in the front line of host defense system against microbial invasions. In this study, we observed a significant decline in CR3–mediated phagocytosis, although the expression of the receptor molecules (CD11b/CD18) and the CR3–induced production of ROI were up–regulated. ROI *per se* are an effective weapon for killing microbes or tumor cells.[21)22)] However, their overproduction is also very harmful to normal cells or tissues,[23)] thereby facilitating the aging process of the body.[28)39)] Although it is impossible to stop the production of ROI, controlling them is essential to prevent an increase in the aging process of both the immune system and the entire body.

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(Received on November 9, 2002,
Accepted on January 9, 2003)