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Expression of CD14, CD16 and CD45RA on monocytes from periodontitis patients

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Background and objective: Peripheral blood monocytes are a heterogeneous population, with phenotypes that change on activation or differentiation. Most of the monocytes express lipopolysaccharide (LPS) receptor, CD14 intensely, and do not express Fc gamma receptor III, CD16 ($CD14^{++}CD16^{-}$ monocytes). But monocytes expressing CD16 with reduced CD14 ($CD14^{+}CD16^{+}$ monocytes) increase in inflammatory diseases as well as sepsis and bacteremia in hemodialysis patients. CD45RA is expressed on activated monocytes, and is regarded as an activation marker of peripheral blood monocytes. The purpose of this study was to determine the phenotypic and functional alteration of monocytes in periodontitis patients.

Methods: Peripheral blood was collected from 33 aggressive periodontitis patients (22 females, 11 males), 55 chronic periodontitis patients (35 females, 20 males) and 30 healthy subjects (16 females, 14 males), and the expression of CD14, CD16 and CD45RA on monocytes was determined using flow cytometry. The production of interleukin-6 (IL-6) by $CD16^{+}$ and $CD16^{-}$ monocytes stimulated with LPS from *Escherichia coli* and *Actinobacillus actinomycetemcomitans* was also examined using flow cytometry.

Results: The percentage of $CD14^{+}CD16^{+}$ monocytes was significantly increased in chronic periodontitis patients. Percentage of monocytes expressing CD45RA was significantly increased in aggressive periodontitis patients compared to healthy subjects. $CD16^{+}$ and $CD16^{-}$ monocytes produced IL-6 in response to LPS from *E. coli* and *A. actinomycetemcomitans*, and the percentage of IL-6 producing cells was higher in $CD16^{+}$ monocytes than $CD16^{-}$ monocytes, suggesting that $CD14^{+}CD16^{+}$ monocytes represent a hyper-reactive phenotype.

Conclusions: The present study demonstrated that $CD14^{+}CD16^{+}$ monocytes and $CD45RA^{+}$ monocytes were increased in chronic and aggressive periodontitis, respectively. These findings suggest that alteration of monocytes in periodontitis patients could be evaluated by monitoring the surface expression of CD14, CD16 and CD45RA on monocytes.

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Periodontitis is an infection of the supporting tissues of teeth. Bacterial plaque harbors up to 1×10^{11} bacteria/g at or below the gingival margin, and a typical tooth with periodontitis can harbor 10^{7}–10^{8} bacteria within a subgingival pocket (1). Subgingival tissues are inflamed and ulcerated, which renders it easy for bacteria or bacterial products, such as lipopolysacchaide (LPS), to enter the circulation. It has been established that bacteremia can be provoked by mastication and oral hygiene procedures, with the extent of bacteremia directly related to the severity of periodontal inflammation (2).

Human monocytes are heterogeneous, and monocyte subsets might be observed in different stages of activation and/or differentiation (3). Most of the monocytes express LPS receptor (CD14) intensely, and do not express Fc gamma receptor III (CD16). Monocyte subsets expressing CD16

with low CD14 ($CD14^+CD16^+$ monocytes) increases in various inflammatory diseases, including septic shock (4) and bacteremia in dialysis patients (5), suggesting that bacteremia caused by periodontitis might affect the expression of CD14 and CD16 in periodontitis patients.

CD45 is a cell surface glycoprotein with a cytoplasmic tyrosine phosphatase domain. Spliced isoforms of CD45 are expressed on a restricted group of cells, and are designated CD45R. CD45RA is the high molecular weight-isoform of CD45 (6). *In vitro* activation of peripheral blood mononuclear cells induces CD45RA expression on monocytes (7), and expression of CD45RA was used as a marker of monocyte activation *in vivo* (8). Examination of CD45RA on monocytes might help to detect the activation of circulating monocytes in periodontitis patients.

Alteration of monocyte subsets in periodontitis patients has not been completely understood. The present study examined the expression of CD14, CD16 and CD45RA on monocytes from chronic and aggressive periodontitis patients as well as healthy subjects in order to determine the relationship between monocyte subsets and periodontitis.

Materials and methods

Subjects

Periodontitis patients were diagnosed as aggressive periodontitis or chronic periodontitis based on the latest classification (9). After obtaining informed consent, 33 aggressive periodontitis patients (22 females and 11 males), 55 chronic periodontitis patients (35 females and 20 males) and 30 healthy subjects (14 males and 16 females) were included in this study. Mean age and standard deviation (SD) for the aggressive periodontitis, chronic periodontitis and healthy subjects were 31 ± 9.9, 55 ± 9.2 and 29 ± 4.2 years, respectively. All patients had generalized periodontitis and the number of teeth showing > 25% bone loss was 31 ± 9.9 in aggressive periodontitis patients and 31 ± 9.9 in chronic periodontitis patients. All subjects were systemically healthy.

Probing pocket depth was measured at six sites per tooth, and measurements were rounded to the nearest 1 mm. Mean pocket depth, and the numbers of teeth showing pocket depth ≥ 3 mm, ≥ 5 mm, and ≥ 7 mm were recorded in the patients and healthy subjects. A set of periapical radiographs was taken for each patient, and bone resorption was examined using Schei's method (10). The number of teeth showing bone loss ≥ 25%, ≥ 50% and ≥ 75% was calculated. Clinical parameters are shown in Table 1.

Cell surface staining

Peripheral blood was collected from the periodontitis patients and healthy subjects, and anticoagulated with heparin at 10 U/ml. One hundred microliters of heparinized whole blood was reacted with either phycoerythrin (PE)-conjugated anti-human CD14 antibody and fluorescein isothiocyanate (FITC) -conjugated anti-CD16 antibody or FITC-conjugated anti-CD45RA antibody (11). FITC- or PE-conjugated isotype control antibodies were added to the control samples.

Cell stimulation and intracellular staining

Flow cytometry and intracytoplasmic staining of IL-6 producing cells were performed to determine which cells produce IL-6 in response to LPS stimulation (12). Whole blood from healthy subjects was stimulated with 1 μg/ml of *Escherichia coli* LPS or *Actinobacillus actinomycetemcomitans* LPS for 6 h at 37°C in 15-ml polypropylene tubes in the presence or absence of 1 mM monensin (GolgiStop, Pharminogen, San Diego, CA, USA). The cultured whole blood cells were stained with FITC-anti-CD16 monoclonal antibody and PerCP-anti-CD14 monoclonal antibody or appropriate isotype control antibodies. The whole blood cells were then incubated for 15 min, and were treated with FACS lysing solution (Becton Dickinson, Mountain View, CA, USA). FACS lysing solution contains less than 5% diethylene glycerol, and less than 1.5% formaldehyde, which lyse red blood cells and fix white blood cells in the whole blood, respectively. After washing with phosphate-buffered saline, cells were permeabilized with saponin (Perm/Wash solution, BD Biosciences) for 5 min at room temperature. For detection of intracellular IL-6, 10 mg/ml PE-conjugated anti-IL-6 antibody was added for 20 min on ice. Samples were then washed twice and re-suspended in phosphate-buffered saline with 0.5% paraformaldehyde for analysis within 4 h.

Statistical analysis

Statistical analysis was performed using Mann–Whitney *U*-test and Spearman's rank correlation test.

Table 1. Clinical parameters of the aggressive and chronic periodontitis patients are shown

	Healthy subjects	Aggressive periodontitis	Chronic periodontitis
Age	29 ± 4.2	31 ± 9.9	55 ± 9.2
Mean pocket depth (mm)	1.0 ± 1.2	3.6 ± 1.6	3.1 ± 0.8
Number of teeth showing			
≥ 3 mm pocket depth	0.0 ± 0.0	21 ± 6.1	20 ± 6.5
≥ 5 mm pocket depth	0.0 ± 0.0	14 ± 8.5	11 ± 6.7
≥ 7 mm pocket depth	0.0 ± 0.0	7.6 ± 6.6	5.3 ± 4.6
≥ 25% bone loss	ND	15 ± 8.1	14 ± 6.9
≥ 50% bone loss	ND	6.0 ± 5.7	5.2 ± 4.2
≥ 75% bone loss	ND	2.1 ± 3.1	1.2 ± 1.6

ND; Not done.
Mean ± SD.

Results

Expression of CD14 molecule on peripheral blood monocytes, lymphocytes and neutrophils

Peripheral blood monocytes, lymphocytes and neutrophils were gated on the basis of forward scatter (FSC) and side scatter (SSC) (Fig. 1a), and expression of CD14 molecule on peripheral blood monocytes (R2), lymphocytes (R1) and neutrophils (R3) was examined. Monocytes were further divided into four subsets (8), based on isotype control staining (Fig. 1b) and CD14 expression. Most of the monocytes (R2) intensely expressed CD14, and did not express CD16 ($CD14^{++}CD16^{-}$ monocytes, Fig. 1c). Some monocytes expressed both CD14 and CD16 intensely ($CD14^{++}CD16^{+}$ monocytes, Fig. 1c). As both $CD14^{++}CD16^{+}$ monocytes and $CD14^{++}CD16^{-}$ monocytes

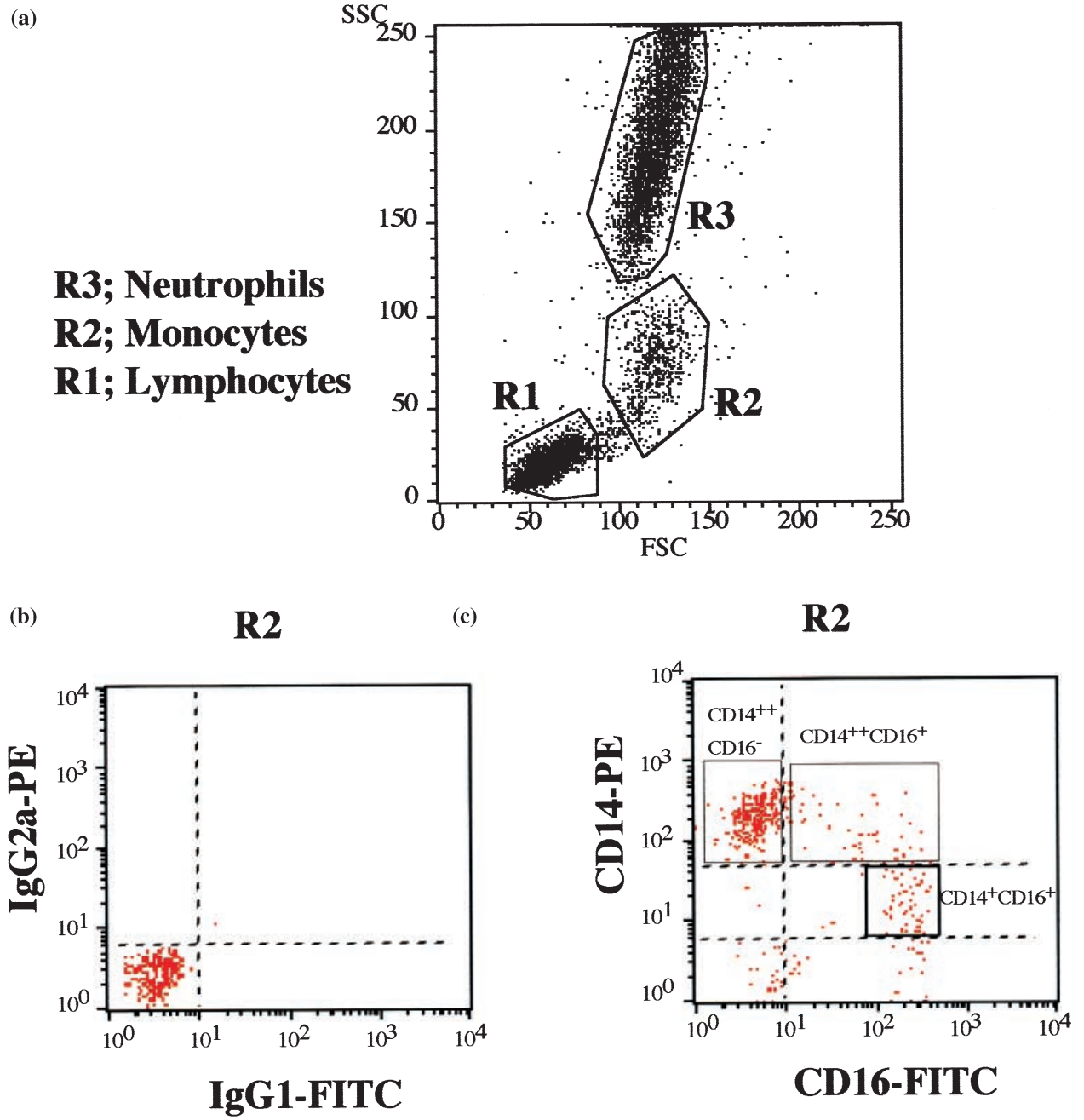

Fig. 1. (a) Peripheral blood was stained with PE-conjugated anti-CD14 and FITC-conjugated anti-CD16 as described in Materials and methods. Monocytes, lymphocytes and neutrophils were gated on the basis of FSC and SSC. FSC and SSC pattern of gated monocytes (R2), lymphocytes (R1) and neutrophils (R3) are shown. (b) Peripheral blood was stained with appropriate isotype control antibody, and FL-1 (FITC) and FL-2 (PE) pattern of gated monocytes (R2) is shown. (c) Expression of CD14 and CD16 on gated peripheral blood monocytes (R2) is shown. Upper horizontal zone; $CD14^{bright}$ monocytes ($CD14^{++}$ monocytes). Middle horizontal zone; $CD14^{dim}$ monocytes ($CD14^{+}$ monocytes). Bottom zone; non-specific PE fluorescence of cells stained with PE conjugated isotype control antibody. Vertical zone on right; $CD16^{+}$ monocytes. Vertical zone on left; non-specific FITC fluorescence.

expressed CD14 intensely, they were regarded as $CD14^{bright}$ monocytes. Some monocytes expressed both CD14 and CD16, but the expression of CD14 was dim ($CD14^{+}CD16^{+}$ monocytes).

The percentages of $CD14^{bright}$ monocytes from periodontitis patients and healthy subjects are shown in Fig. 2. The percentage of $CD14^{bright}$ monocytes in chronic periodontitis patients was significantly lower than healthy subjects (healthy subjects; 82 ± 1.7%, chronic periodontitis patients; 70 ± 2.5%, $p < 0.05$, Mann–Whitney *U*-test). The mean percentage of $CD14^{bright}$ monocytes in aggressive periodontitis patients (70 ± 2.5%) was also lower than healthy subjects, but the difference was not statistically significant.

Percentages of $CD14^{++}CD16^{-}$ monocytes were not significantly different among aggressive periodontitis, chronic periodontitis and healthy subjects (healthy subjects, 59 ± 5.0%; chronic periodontitis, 49 ± 4.0%; aggressive periodontitis, 57 ± 5.1%). Percentages of $CD14^{++}CD16^{+}$ monocytes were not significantly different among aggressive periodontitis, chronic periodontitis and healthy subjects (healthy subjects, 23 ± 5.3%; chronic periodontitis, 20 ± 3.2%; aggressive periodontitis, 20 ± 4.0%).

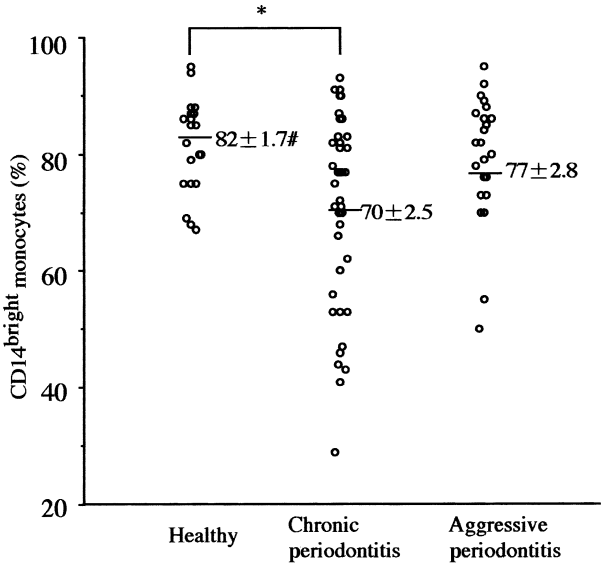

Fig. 2. Peripheral blood was stained with PE-conjugated anti-CD14, and percentages of CD14 bright population in monocytes from aggressive and chronic periodontitis patients and healthy subjects are shown. *$p < 0.05$, Mann–Whitney *U*-test; mean ± standard error.

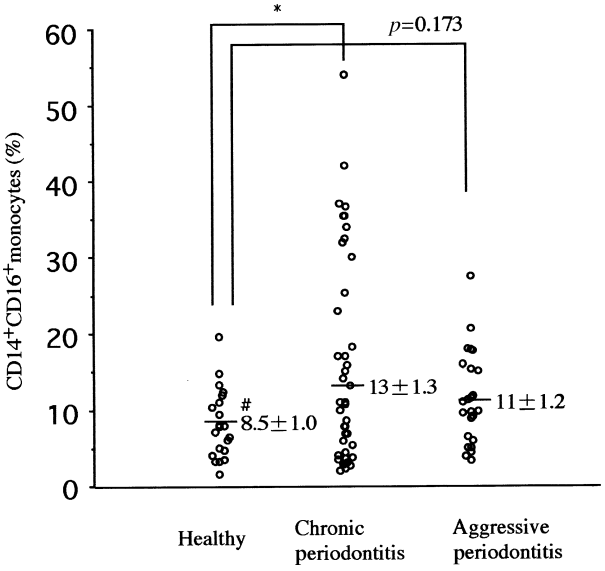

Fig. 3. Peripheral blood was stained with PE-conjugated anti-CD14, and percentages of $CD14^{+}CD16^{+}$ monocytes in aggressive and chronic periodontitis patients and healthy subjects are shown. *$p < 0.05$, Mann–Whitney *U*-test; mean ± standard error.

Increase of $CD14^{+}CD16^{+}$ monocytes in chronic periodontitis patients

Percentages of $CD14^{+}CD16^{+}$ monocytes from periodontitis patients and healthy subjects are shown in Fig. 3. The percentage of $CD14^{+}CD16^{+}$ monocytes in chronic periodontitis patients was significantly higher than in healthy subjects (healthy subjects, 8.5 ± 1.0%; chronic periodontitis patients, 13 ± 1.3%; $p < 0.05$, Mann–Whitney *U*-test). Mean percentage of $CD14^{+}CD16^{+}$ monocytes in aggressive periodontitis patients (11 ± 1.2%) was also higher than healthy subjects, but the difference was not statistically significant. Percentage of $CD14^{bright}$ monocytes was negatively correlated to the percentages of $CD14^{+}CD16^{+}$ monocytes in healthy subjects, chronic periodontitis patients and aggressive periodontitis patients to a significant level ($p < 0.05$, Spearman's rank correlation test).

Subjects with the median percentage of $CD14^{bright}$ monocytes in healthy, chronic and aggressive periodontitis patients were selected, and the expression of CD14 and CD16 on monocytes in these subjects is shown in Fig. 4. As expected, the majority of monocytes were $CD14^{++}CD16^{-}$ monocytes. The percentage of $CD14^{+}CD16^{+}$ monocytes was highest in chronic periodontitis patients (15.5%), followed by aggressive periodontitis patients (9.5%) and then by healthy subjects (7.1%).

Increase of $CD45RA^{+}$ monocytes in aggressive periodontitis patients

The percentages of $CD45RA^{+}$ monocytes from periodontitis patients and

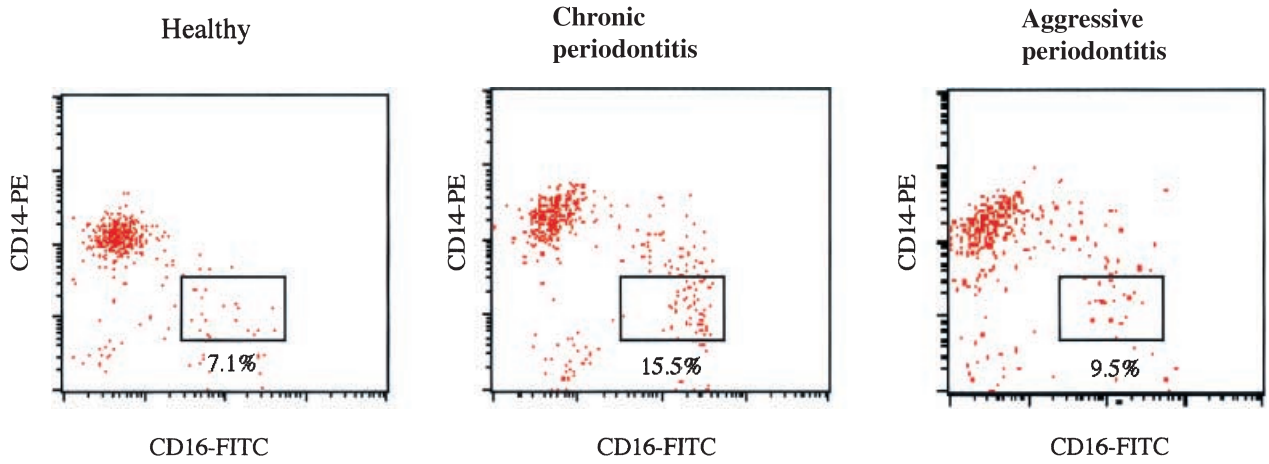

Fig. 4. Subjects with the median percentages of $CD14^{bright}$ monocytes in healthy, chronic and aggressive periodontitis patients were selected, and expression of CD14 and CD16 on gated monocytes is shown. Open square; $CD14^+CD16^+$ monocytes.

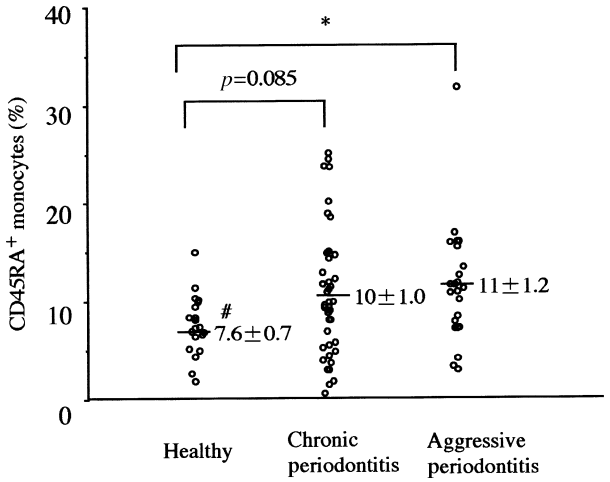

Fig. 5. Peripheral blood was stained with PE-conjugated anti-CD45RA, and percentages of $CD45RA^+$ monocytes in aggressive and chronic periodontitis patients and healthy subjects are shown. *$p < 0.05$, Mann–Whitney *U*-test; mean ± standard error.

healthy subjects are shown in Fig. 5. The percentage of $CD45RA^+$ monocytes in aggressive periodontitis patients was significantly higher than healthy subjects (healthy subjects, 7.6 ± 0.7%; aggressive periodontitis patients, 11 ± 1.2%, $p < 0.05$, Mann–Whitney *U*-test). Mean percentage of $CD45RA^+$ monocytes in chronic periodontitis patients (10 ± 1.0%) was also higher than healthy subjects, but the difference was not statistically significant ($p = 0.085$, Mann–Whitney *U*-test).

Percentages of $CD14^+CD16^+$ monocytes and $CD45RA^+$ monocytes were significantly correlated in aggressive periodontitis patients ($p < 0.05$, Mann–Whitney *U*-test), whereas this correlation was not observed in chronic periodontitis.

Production of IL-6 by $CD14^{++}CD16^-$ and $CD14^+CD16^+$ monocytes in whole blood cultured with LPS from *E. coli* and *A. actinomycetemcomitans*.

Whole blood cells were cultured with or without *E. coli*, and *A. actinomycetemcomitans* LPS, and cells were stained with FITC anti-CD16 and peridinin chlorophyll protein (PerCP) anti-CD14 antibodies, followed by PE conjugated anti-IL-6 antibody as described in the Materials and methods section. $CD14^+$ cells were gated (Fig. 6a), and expression of CD16 and IL-6 was analyzed. Both $CD16^-$ and $CD16^+$ monocytes expressed IL-6 in response to stimulation with LPS from *E. coli* and *A. actinomycetemcomitans*. Although more than 80% of $CD16^+$ monocytes expressed cytoplasmic IL-6, only about 60% of $CD16^-$ monocytes expressed IL-6 (Fig. 6b). Interestingly, part of the $CD16^+$ monocytes expressed IL-6 without stimulation, whereas $CD16^-$ monocyte did not express IL-6 without stimulation (Fig. 6b).

Discussion

$CD14^+CD16^+$ monocytes have been reported to increase in bacteremia (4, 5). In this study, $CD14^+CD16^+$ monocytes were increased in chronic periodontitis patients. Since the majority of monocytes do not express CD16 (13), and tissue macrophages express CD16, $CD14^+CD16^+$ monocytes are regarded to be more mature than $CD14^{++}CD16^-$ monocytes. This was supported by *in vitro* maturation studies, which revealed that $CD14^+CD16^+$ monocytes could be generated from $CD14^{++}CD16^-$ peripheral blood monocytes (14–16).

It has been reported that $CD14^+CD16^+$ monocytes were induced after repeated injection of LPS (17). Similarly, macrophage colony-stimulating factor (M-CSF) treatment induced $CD14^+CD16^+$ monocytes, and the effect was enhanced by combined M-CSF/interferon-γ injection (18). The increase of $CD14^+CD16^+$ monocytes in chronic periodontitis patients suggests that bacteremia caused by periodontitis might induce cytokines to

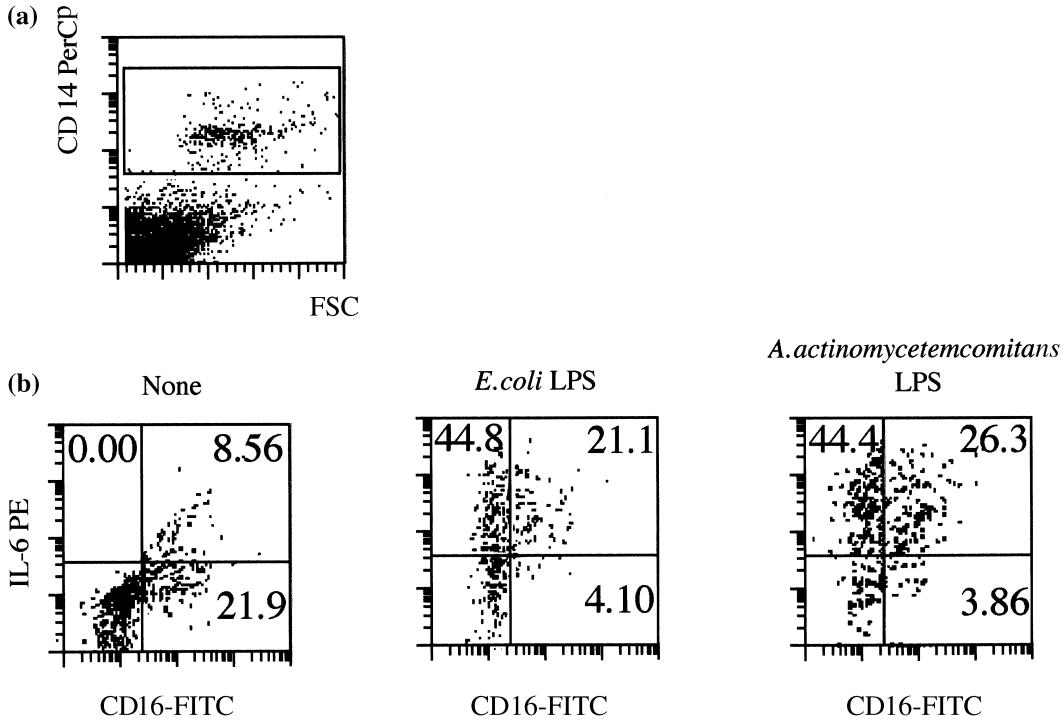

Fig. 6. Intracytoplasmic IL-6 staining of peripheral blood monocytes stimulated with *E. coli* and *A. actinomycetemcomitans* LPS are shown. Peripheral blood from healthy subjects was stimulated with or without LPS, and cultured in the presence or absence of monensin for 6 h. Cultured cells were stained with PE-conjugated anti-IL-6 antibody. Specificity of the IL-6 staining was verified by neutralization with unlabelled anti-IL-6 antibody. Representative results from three experiments are shown. (a) The gate for $CD14^+$ monocytes. (b) The expression of CD16 and IL-6 in $CD14^+$ monocytes.

develop $CD14^+CD16^+$ monocytes. Alternatively, cytokines released from a periodontal lesion might activate $CD14^{++}CD16^-$ monocytes to differentiate into $CD14^+CD16^+$ monocytes.

Belge *et al.* reported that $CD14^+CD16^+$ monocytes are mainly involved in the production of tumor necrosis factor-alpha (TNF-α) compared to $CD14^{++}CD16^-$ monocytes (19). Our results support their findings, as $CD14^+CD16^+$ monocytes produced more IL-6 than $CD16^-$ monocytes. Periodontitis patients with increased $CD14^+CD16^+$ monocytes might produce IL-6 in response to bacteremia. Several investigators reported that levels of C-reactive protein are increased in periodontitis (20, 21). As IL-6 is known to induce production of C-reactive protein in the liver (22), increased C-reactive protein in periodontitis patients might be related to the IL-6 production by monocytes during bacteremia.

The activation-associated expression of CD45RA, which is another marker of monocyte heterogeneity, was increased in aggressive periodontitis patients. As CD45RA is mainly expressed on $CD14^+CD16^+$ monocytes (8, 23), $CD45RA^+$ monocytes might be an activated subset of $CD14^+CD16^+$ monocytes. The percentages of $CD14^+CD16^+$ monocytes and $CD45RA^+$ monocytes were significantly correlated in aggressive periodontitis patients, although this correlation was not observed in chronic periodontitis patients. Different mechanisms might control the differentiation of monocyte subsets in aggressive and chronic periodontitis, in addition to genetic factors and periodontal infection.

Beck *et al.* demonstrated that certain forms of periodontal disease, including early onset and refractory periodontitis, possess hyper-inflammatory monocytes that might be genetically determined (24). They proposed an association of hyper-inflammatory monocytes with both periodontal disease and atherosclerosis (24). Losche *et al.* reported that total cholesterol and atherogenic low-density lipoprotein cholesterol were significantly higher in periodontitis patients compared to age- and sex-matched control subjects, even though both groups were systemically healthy according to the medical history (25). Polymorphisms of the genes that affect both monocyte differentiation and atherosclerosis might be a genetic risk factor for periodontitis associated with the alteration of monocyte subsets.

Apolipoprotein E (apoE) is a molecule that affects both monocyte differentiation and atherosclerosis. In humans, three major isoforms of apoE designated as apoE2, apoE3 and apoE4, which are products of three alleles at a single gene locus (26), and apoE4 phenotype have been shown to be a risk factor for atherosclerosis (27). Rothe *et al.* reported that $CD14^+CD16^+$ and $CD45RA^+$ monocytes are positively correlated to total cholesterol and low-density-lipoprotein, respectively (8). They suggested

that $CD45RA^+$ expression is an indicator of monocyte activation in the presence of atherogenic lipoproteins. $CD14^+CD16^+$ monocytes are increased in correlation to apoE4 allele (8), and apoE4 phenotype significantly influences the M-CSF-dependent differentiation of monocytes toward a $CD16^+$ phenotype (28). Increase of $CD45RA^+$ monocytes in aggressive periodontitis patients in this study might be related to genetic factors including apoE, and further studies are necessary to clarify the association between periodontitis and genetic factors that regulate both monocyte differentiation and atherosclerosis. Considering that the diagnosis of aggressive periodontitis was based on clinical findings, and $CD45RA^+$ monocytes were not increased in all aggressive periodontitis patients, there might be a type of aggressive periodontitis associated with increased CD45RA on monocytes.

In conclusion, $CD14^+CD16^+$ and $CD45RA^+$ monocytes were increased in chronic and aggressive periodontitis, respectively. Alteration of peripheral blood monocytes in periodontitis patients might be monitored by surface expression of CD14, CD16 and CD45RA on circulating monocytes. It remains to be determined whether these alterations were genetically determined or induced by the periodontitis.

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