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フローサイトメトリーによるヒト唾液好中球の評価(A Flow Cytometric Analysis for Evaluation of Human Salivary Neutrophils)

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（本文39～43ページ）

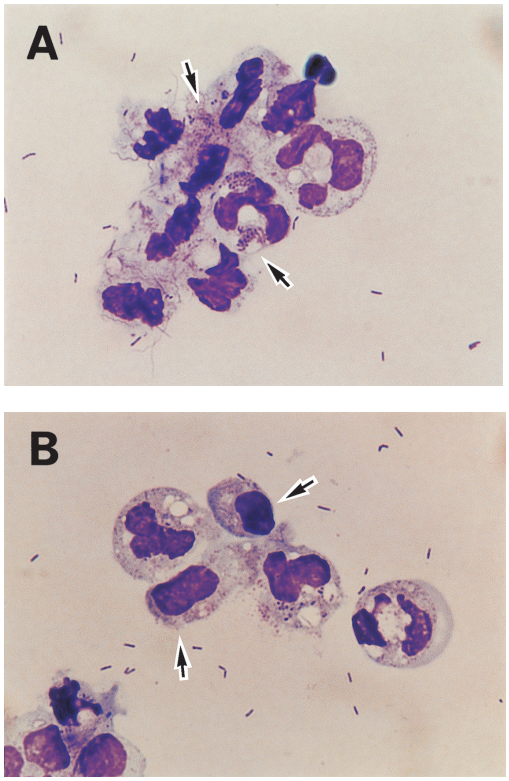

Fig. 1. Microscopic photographs of salivary leukocytes stained with May-Grunwald and Giemsa solution. The arrows in panel A indicate segmented neutrophils, and those in panel B indicate immature juvenile neutrophils with a rod nucleus. The upper cell in panel C is an epithelial cell.

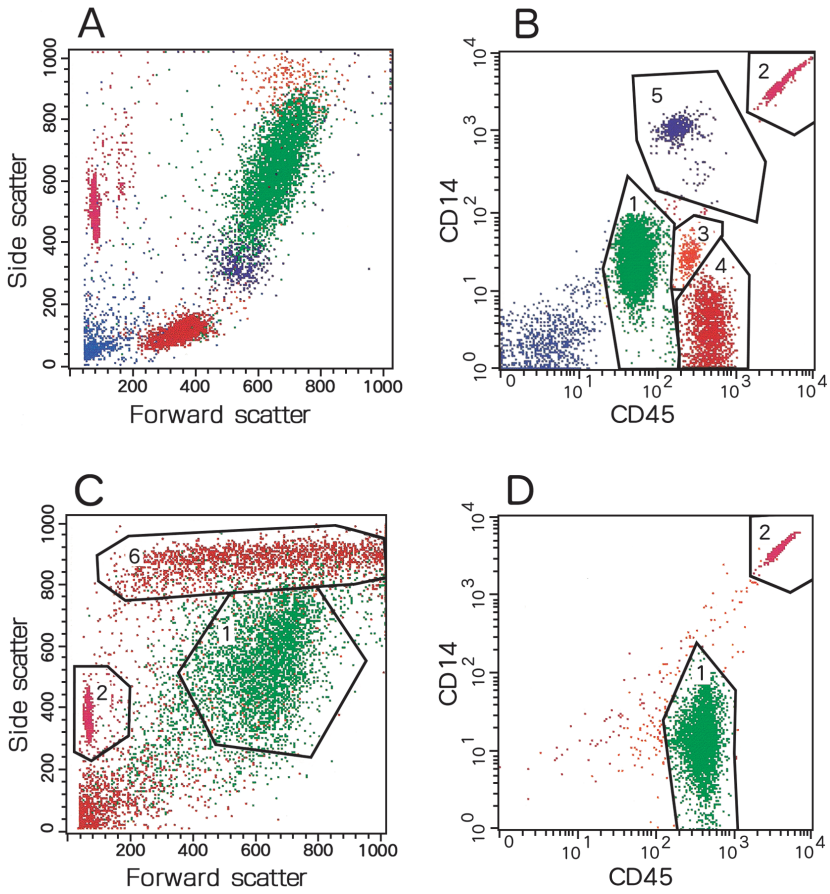

Fig. 2. Flow cytometric analysis of salivary leukocytes and peripheral blood leukocytes. A and C represent forward scatter (FSC) and side scatter (SSC) dot plots of peripheral blood leukocytes (A) and salivary cells (C), respectively. B and D represent CD45 vs. CD14 two-color dot plots of peripheral blood leukocytes (B) and salivary leukocytes (D). The number of 1-6 in panels B-D indicate neutrophils (1), beads of TruCountTM (Becton Dickinson), eosinophils (3), and lymphocytes (4), monocytes (5) and epithelial cells (6). One representative example is shown with reproducible observation of three independent experiments for three volunteers.

投稿論文（原著）

フローサイトメトリーによるヒト唾液好中球の評価

永　井　伸　夫*、林　　要喜知**、山　田　幸　宏*

【要　　旨】

ヒト唾液中に含まれる白血球細胞組成を調べる為に、フローサイトメトリーによる解析を行なった。ボランティアから得た新鮮な唾液サンプルを用いて解析を行なったところ、99％以上が好中球であることが判明した。単球やリンパ球は、解析したどのボランティアのサンプルの場合でも、検出できなかった。唾液中１μℓ当たりの好中球数は、平均6409±5816と推定されたが、個人差が大きかった。この単純かつ非侵襲的な方法は、看護、口腔ケア、心理学、あるいは、食品科学などの分野において、健康状態やストレス評価のための有益な手段となるであろう。

キーワード　唾液，好中球，フローサイトメトリー，　ヘルスアセスメント

INTRODUCTION

Human neutrophils play crucial roles both in protecting hosts against invading microbes and in immunologically induced acute tissue injuries[1]. When neutrophils ingest particles or are exposed to stimuli in the form of soluble substances, they exert a number of specific functions such as chemotaxis[2], cell adhesion[3, 4], phagocytosis[5], and secretion of granule contents[6].

The leukocytes in the oral cavity are known to be gingival, creviced and salivary leukocytes[7, 8]. However, few studies have been carried out to examine the physiological roles or flow cytometric analysis of salivary leukocytes.

In this short report, we describe a simple flow cytometric method for isolation and analysis of human salivary leukocytes.

MATERIALS AND METHODS

Human salivary leukocytes were obtained by a modification of the method of Yamamoto et al.[9]. Oral cavities of three healthy volunteers (21-22 years of age) were thoroughly washed with 15 ml of Ca^{2+} - Mg^{2+} - free Hank's balanced salt solution (HBSS) for 30 s. The oral washings (100 ml) were centrifuged at 250 x g for 5 min. The precipitated cells were suspended in HBSS and passed in sequence through nylon sheets (ASTM 200-74, Nytal, Switzerland). The effluent was then centrifuged again at 250 x g for 5 min, and the resultant pellet was resuspended in HBSS. After counting cell numbers, each cell suspension was diluted in phosphate-buffered saline (PBS, pH 7.0; Dako, Carpinteria, CA, USA) containing 2% (w/v) fetal bovine serum (FBS; Nippon-biotest, Tokyo) to make cell suspension at a concentration of 1×10^5 - 1×10^6 cells/ml. Cell viability was defined as those that excluded 0.05 % trypan blue in PBS with 0.1 % bovine serum albumin (BSA; Nacalai, Kyoto). Two-color immunofluorescent staining for flow cytometric analysis of salivary leukocytes was performed by the method of Terstappen et al.[10]. Briefly, 50 μl of sample aliquots were stained with 20 μl

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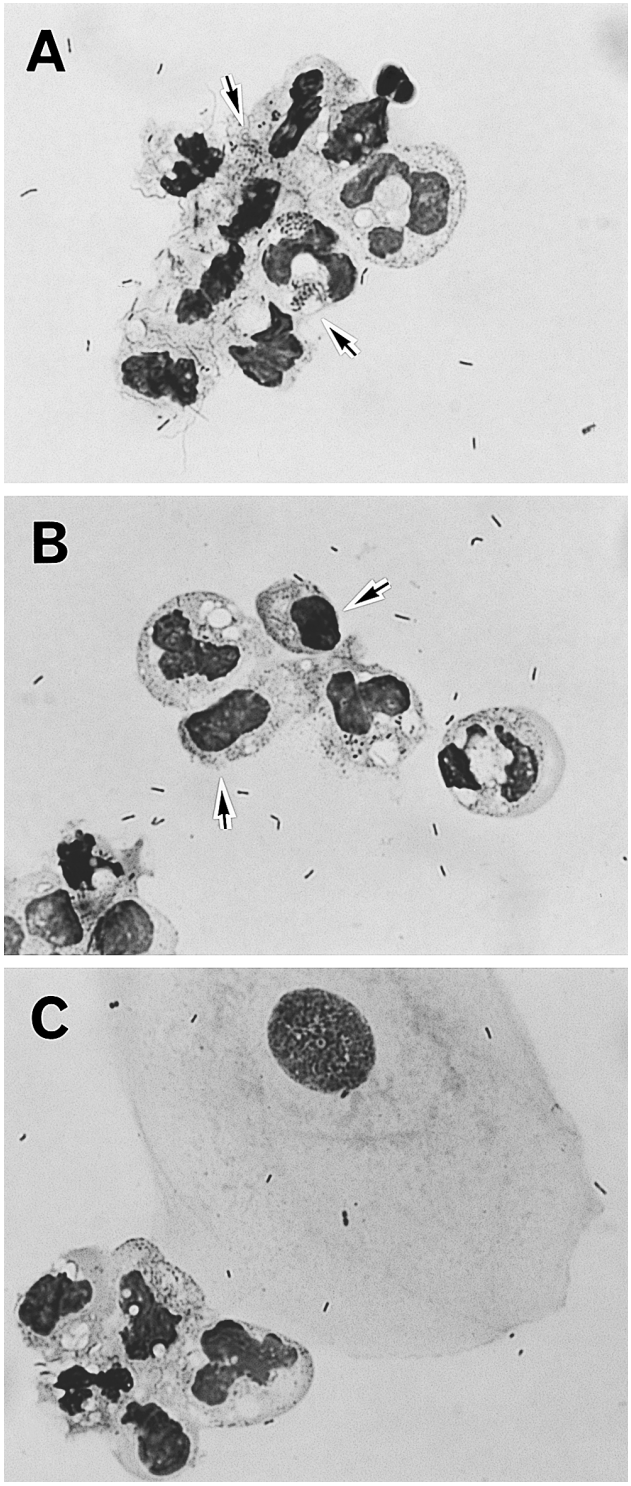
of the staining antibody combination including both CD45 conjugated with fluorescein isothiocyanate (FITC) and CD14 conjugated with phycoerythrin (PE) (LeucoGATE™, Becton Dickinson, San Jose, CA, USA). IgG isotype control antibody conjugates were analyzed to establish background fluorescence. The samples were analyzed using a FACScan™ flow cytometer (Becton Dickinson), and the acquired data were analyzed using a CELL Quest™ software (Becton Dickinson). The absolute neutrophil cell count per one μl of saliva was calculated as follows:

$$\text{Abs. cell count (cells/}\mu\text{l)} = \frac{\text{cell count of flow cytometric measurement on a neutrophil's region (e.g. Fig.2D, region1)} \times \text{a constant number (total beads count) of TruCount ™ (Becton Dickinson)}}{\text{beads count of flow cytometric measurement on a beads' region (e.g. Fig.2D, region2)}} \times \frac{1}{\text{sample volume (}\mu\text{l)}} \times \text{dilution rate}$$

RESULTS AND DISCUSSION

The leukocytes prepared from the oral cavity were stained with May-Grunwald and Giemsa solution. This morphological analysis revealed that these oral cells were either neutrophils or epithelial cells (Fig.1). More than 80 % of the neutrophils were segmented ones with 2-3 lobed nuclei, and the rest were more immature or juvenile neutrophils. The salivary neutrophils appeared to be similar to typical human peripheral neutrophils when compared under similar experimental conditions. In the samples, neither monocytes nor lymphocytes were observed at all.

To confirm the results, we next performed cytometric analysis to compare the salivary leukocytes and peripheral blood leukocytes using a two-color immunofluorescent staining for cytometric analysis (Fig. 2). The salivary leukocytes showed a different pattern from that of peripheral blood leukocytes under analyses of forward scatter (FSC) and side scatter (SSC) (Fig. 2A and Fig. 2C). Analysis using the antibodies CD45 and CD14, which are specific makers for all human leukocytes (11) and human monocytes (12) respectively, clearly identified the population of salivary leukocytes; moreover the salivary leukocytes and peripheral blood leukocytes each produced distinctive patterns (Fig. 2B and D). In the region where leukocytes exist, more than 99 % of the salivary cells were



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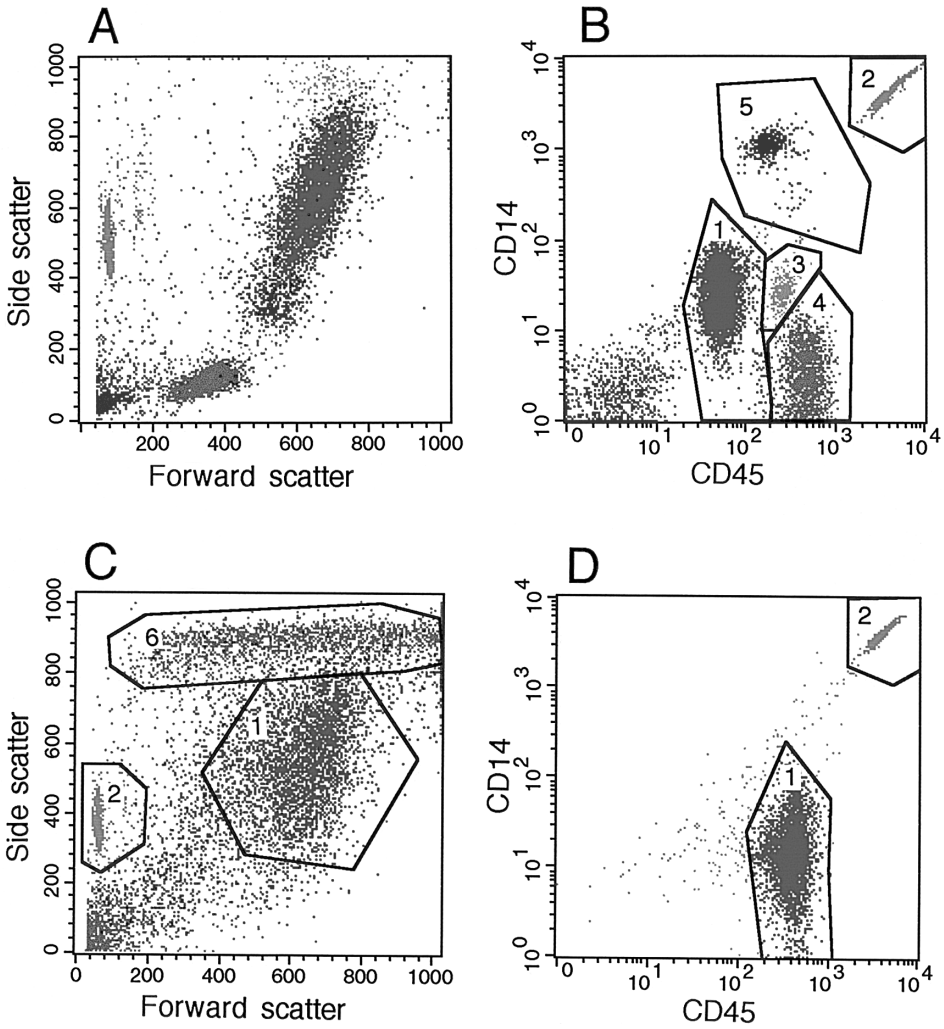

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found to be neutrophils. Neither monocytes nor lymphocytes were detected, as was expected. The cell number of salivary neutrophils from three healthy volunteers was 6409 ±5816 (mean±S.D.) / ml under flow cytometric analysis (Table 1). This observation is consistent with that in Fig 1 as well as the previous report (9). Therefore, we concluded that more than 99% of the salivary leukocytes were neutrophils.

The oral cavity is an important area of primary defense against viruses, bacteria, fungi, protozoa and other microbes[1]. The phagocytic neutrophils are known to migrate constantly into the oral cavity[7,8]. Interestingly, the neutrophils we found in the saliva were more differentiated and mature than those in the peripheral blood. If this primary defense system is weakened for some reason such as poor general health, stress, or disease, there can be serious consequences especially in the old and in people under long-term nursing care. It is, therefore, important to assess the activities of the oral primary defense in the more vulnerable groups, possibly including patients of mental and psychological disorders, too, in order to gain information relating to health, disease and nursing care.

TABLE.1 The absolute cell count of salivery neutrophils from three healthy volunteers analyxed by flow cytometry

volunteer	expt.1 (cells/ml saliva)	expt.2 (cells/ml saliva)	mean of twice expts. data (cells/ml saliva)
No. 1	12320	13909	13114
No. 2	1165	4287	2726
No. 3	4442	2333	3387
mean＋S.D.	5976±5733	6843±6197	6409±5816

To date, application of this flow cytometric method to evaluate the salivary leukocytes has not been done. If we could both quantify and qualify the activity of oral neutrophils, it will provide a new method for health assessment involved in nursing, oral care, psychology and food science[13,14,15]. Further investigation of the phagocytic activities and the cell surface antigens of salivary neutrophils will be required to clarify their biological reaction mechanism and to realize the potential applications.

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A Flow Cytometric Analysis for Evaluation of Human Salivary Neutrophils

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Summary

To examine the cellular population of lukocytes in human saliva, we employed a simple flow cytometric method of analysis. Using this system with freshly prepared samples from young volunteers, we found that more than 99% of the salivary leukocytes turned out to be neutrophils. Neither monocytes nor lymphocytes were detectable in any volunteer specimen examined. The cell number of salivary neutrophils was estimated to be 6409±5816 (mean±S.D.) /ml with a large variation among the volunteers. This simple and noninvasive method will provide a useful method for assessment of health and stress in nursing, oral care, psychology and food science .

Key words saliva, neutrophils, flow cytometry, health assessment

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