A New Method for Isolation of Salivary Neutrophils and Determination of Their Functional Activity

M. ASHKENAZI and D.K. DENNISON[1]

Department of Periodontics, Dental Branch, University of Texas Health Science Center-Houston, 6516 John Freeman Avenue, Houston, Texas 77025

The purposes of this study were to develop a new method for isolating salivary neutrophils (SPMNs), and to determine their functional activity. Studies of neutrophils in the oral cavity have been largely limited to crevicular PMNs, because of difficulties in obtaining viable SPMNs free of epithelial cells. A method to obtain SPMNs is presented. Donors performed rapid sequential rinsings by placing in their mouths 15 mL of Hanks' balanced salt solution [free of calcium or magnesium ions (HBSS-CMF)], which contained 0.1% gelatin, then swishing the solution for 30 s, and expectorating into a polypropylene receptacle containing 400 mL 4°C HBSS-CMF. This sequence was repeated for 20 min. The collected solution was stirred for 10 min, the cells were washed, and the re-suspended pellet was passed sequentially through a 20-μm and a 10-μm nylon mesh. The cells consisted of 97.7 ± 1.7% SPMNs, only 2.3% epithelial cells, and were almost free of oral debris.

The SPMNs were studied for CD11b expression, H_2O_2 production, and F-actin polymerization. SPMNs had significantly higher resting values for CD11b, H_2O_2 production, and F-actin polymerization compared with blood neutrophils (BPMNs). SPMNs responded to stimulation by chemotactic peptide or phorbol ester in a dose-dependent fashion, with levels of CD11b, H_2O_2, and F-actin comparable with BPMNs at optimal stimulant concentrations. The elevated resting levels of CD11b, H_2O_2, and F-actin for SPMNs were probably caused by exposure to gingival and oral bacteria. The ability of in vitro-*stimulated SPMNs to increase their levels of CD11b, H_2O_2, and F-actin to levels comparable with BPMNs indicates that these cells can function after leaving the gingival crevices. The method described is relatively simple and provides a high number (4-30 × 10^6) of purified and functional SPMNs.*

J Dent Res 68(8):1256–1261, August, 1989

Introduction.

Polymorphonuclear leukocytes (PMNs) play a major role in host defense against infections. Large numbers of PMNs constantly migrate from the blood through the gingival crevice into the oral cavity. Indeed, systemic disturbances in number or function of peripheral blood PMNs (BPMNs) commonly result in significant oral complications (reviewed by Van Dyke, 1985, and Van Dyke *et al.*, 1985). The PMNs in the oral cavity can be divided into two major groups: (1) gingival or crevicular PMNs (GPMNs), which are found in the gingival crevice, before migrating into the oral cavity (Sharry and Krasse, 1960); and (2) salivary PMNs (SPMNs), which are found in saliva, and originate mainly in gingival crevices (Schiott and Löe, 1970). GPMNs have been comprehensively investigated, and their role in periodontal health and disease is well-established (Miller *et al.*, 1984; Fine and Mandel, 1986; Cohen *et al.*, 1985; Genco and Slots, 1984). Our knowledge of SPMNs is limited, and controversies exist with regard to their function and significance. For example, studies that measured the oral migratory rate (OMR) of SPMNs showed conflicting results: Wright (1962) found lower OMR in edentulous adults than in dentulous adults, while Raeste (1976) found no such difference. The number of SPMNs in a given individual generally correlates with gingivitis, but the OMR does not necessarily reflect the degree of clinical gingivitis (Schiott and Löe, 1970). In contrast, no correlation was found between OMR and the degree of gingival inflammation in children (Cox *et al.*, 1974).

The reported viability of isolated SPMNs has been very low (Sharry and Krasse, 1960; Scully, 1982). Functionally, Scully (1982) found reduced phagocytic activity of SPMNs, compared with that in blood and gingival PMNs. In contrast, Sela *et al.* (1981) found that a significantly higher percent of SPMNs than BPMNs could phagocytose and bind *via* complement receptors. The main reason for the limited data on SPMNs is the difficulty in obtaining a relatively high number of viable cells, free of contaminating epithelial cells, bacteria, and oral debris (Klinkhamer, 1963).

The importance of SPMNs in protecting the oral mucosa against infection is unclear. SPMNs are usually regarded as non-functional, post-inflammatory cells. However, their presence in high numbers in the oral cavity might play a protective role, by preventing oral and periodontal diseases, including ulcerations. Functional studies of SPMNs can elucidate their ability to prevent oral diseases, and may allow for correlation of their function with disease states. Furthermore, alterations in the functional activity of SPMNs in oral diseases may provide a simple method to evaluate exacerbation or progression of the disease process.

The aims of this study were to develop a new method to isolate SPMNs, and to determine their functional activity. In this study, we evaluated several functional activities of SPMNs, including: H_2O_2 production as a measure of the bactericidal activity of the cells; CD11b expression, which correlates with the ability of PMNs to adhere to and ingest bacteria; and F-actin polymerization, which is an obligatory event for leukocyte motility. We compared the results with those for BPMNs.

Materials and methods.

Salivary and blood PMNs were obtained simultaneously from healthy donors, and were tested on the same day for functional activity.

Isolation of SPMNs.—We examined seven donors (ages 28-37 years) with minimal or no periodontal inflammation; we examined two donors twice. We obtained viable SPMNs from donors by repeated rinsing of the oral cavity, and isolated the SPMNs from oral epithelial cells by using nylon mesh. Donors placed in their mouths 15 mL of Hanks' balanced salt solution [free of Ca^{2+} or Mg^{2+} (HBSS-CMF, GIBCO Laboratories, Grand Island, NY; at pH 7.4)] containing 0.1% gelatin at 24°C, swished the solution for 30 s, and expectorated into a polypropylene vessel containing 400 mL 4°C HBSS-CMF. This sequence was repeated without interruption for 20 min (about 40 cycles).

Received for publication January 30, 1989
Accepted for publication April 17, 1989
[1]To whom correspondence and reprint requests should be addressed
This investigation was supported by research grant DE-7502 from the National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892.

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The collected solution was stirred (10 min) and centrifuged (5 min at 250 g), and the re-suspended pellet was passed sequentially through a 20-μm nylon mesh and a 10-μm nylon mesh. This procedure separated the salivary PMNs from oral epithelial cells and debris. The effluent containing the SPMNs was centrifuged (5 min, 250 g), and the pellet re-suspended in HBSS containing Ca^{2+} and Mg^{2+} (HBSS) but no gelatin. Using a Coulter Counter (model ZBI, Coulter Electronics, Inc., Hialeah, FL), we then counted the cells and re-suspended them at a concentration of 3.3×10^6 cells/mL. Viability was determined by trypan blue exclusion (Fallon *et al.*, 1962). The purity of the SPMNs was determined objectively by direct observation with a light microscope, and subjectively by use of the flow cytometer.

We also separated SPMNs by previously described methods (Wilton *et al.*, 1977a; Sela *et al.*, 1981) using density gradients (fetal calf serum and Ficoll Triosil). Briefly, the donors washed their mouths with 15 mL of HBSS-CMF containing 0.1% gelatin for 30 s, and expectorated into 50-mL polypropylene test tubes. This sequence was repeated for 5 min. The collected solution was pooled and centrifuged for 10 min at 250 g. The cell pellet was pooled in 2 mL of HBSS-CMF, carefully layered on 1 mL of fetal calf serum (FCS; GIBCO, Grand Island, NY), and centrifuged at 100 g for 10 min. The pellet was washed twice in cold HBSS-CMF, re-suspended, and spun onto slides. Slides were stained with Diff-Quik (American Scientific Products, McGaw Park, IL) for microscopic evaluation. Furthermore, cells obtained after centrifugation through FCS were layered on a 1-mL Ficoll-Triosil gradient ($\alpha = 1.077$ g/cm^3), centrifuged at 450 g for 20 min, washed with HBSS-CMF, and evaluated microscopically.

Isolation of human blood PMNs.—Blood from donors was collected by venipuncture in 10-mL vacutainers (Becton Dickinson, Rutherford, NJ) that contained 0.1 mL of 15% EDTA. Ten mL of blood was mixed with 1.5 mL of 6% dextran (M.W. 229,000; Sigma, St. Louis, MO) in HBSS-CMF, and allowed to sediment at room temperature for 30 min. The supernatant—or leukocyte-rich plasma—was washed twice with ice-cold HBSS-CMF containing 0.1% gelatin, and centrifuged (250 g, 5 min) to obtain BPMNs. BPMNs were re-suspended in ice-cold HBSS without gelatin at a final concentration of 3.3×10^6 cells/mL.

PMNs analyses.—We measured samples of SPMNs and BPMNs using an Ortho Cytofluorograf System 50-H with a 2150 computer (flow cytometer, Ortho Diagnostics, Westwood, MA). We illuminated the cells with a 5-watt argon laser emitting 400 milliwatts of light at 488 nm. Using forward and right-angle light scatters, we selected PMNs to be analyzed for fluorescence. A green fluorescence histogram was collected for 10,000 PMNs for each sample analyzed. As shown in Figs. 1a and 1b, a histogram of forward/right-angle scatters was used. The right-angle scatter is related to the internal structure of the cell, while the forward light scatter is related to the general size of the cell. The size, the multi-lobed nuclei, and the numerous granules of PMNs allow for discrimination from lymphocytes, epithelial cells, and oral debris on the flow cytometer. Thus, PMNs were identified as having more right-angle and forward scatters than lymphocytes, but less forward scatter than epithelial cells.

Oxidative burst development (H_2O_2 production).—The functional activity of SPMNs was evaluated by stimulating the cells and measuring H_2O_2 as a product of the oxidative respiratory burst. After stimulating the cells with phorbol myristate acetate (PMA) or N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, Sigma), we determined H_2O_2 production using the fluorescent probe dichlorofluorescein (DCF*), as described by Bass *et al.* (1983). Briefly, we "loaded" SPMNs and BPMNs suspensions (300 μL, 3.3×10^6 cells/mL) with 2'7'dichlorofluorescein diacetate (DCF-DA, Kodak Chemicals, Rochester, NY), at a concentration of 90 μg DCF-DA/mL (15 min, 37°C). [Loading is accomplished when the non-polar DCF-DA enters the cell, and the diacetate group is cleaved by intracellular esterases, trapping the non-fluorescent polar dichlorofluorescein (DCF) within the cell.] We then stimulated the cells (15 min, 37°C) with PMA (2 or 20 μg/mL) or FMLP (final concentration: 10^{-9} mol/L, 10^{-7} mol/L, 10^{-5} mol/L) in a shaking water bath. Controls loaded with DCF were incubated with HBSS at 37°C or on ice. We stopped both reactions by placing the cells on ice. Intracellular production of H_2O_2, which caused hydrolysis of DCF to the fluorescent analog DCF*, was measured within two hours by flow cytometric analysis.

Expression of CD11b/CD18 adhesion receptors.—We determined CD11b/CD18 expression using monoclonal antibody specific for the α chain (CD11b) of the CD11b/CD18 adhesion glycoprotein complex (Anderson *et al.*, 1985). Briefly, we incubated, in a shaking water bath, 300 μL of BPMNs suspension (3.3×10^6 cells/mL) with the stimulant FMLP (final concentration of 10^{-6} mol/L, 10^{-7} mol/L, 10^{-9} mol/L) or HBSS (control), for 15 min at 37°C. We placed a parallel non-stimulated sample on ice. After incubation, we stopped the reaction by placing the tubes on ice for two min. We then performed the remainder of the experiment using ice-cold solutions and incubation on ice to prevent further mobilization of CD11/CD18. Cell suspensions were washed (centrifugation: 2000 g, 2 min), re-suspended in 100 μL HBSS, and incubated for 30 min with 1 μg OKM-1 (Ortho Diagnostics, Raritan, NJ; Murine monoclonal antibody to the CD11b; CD11b is the α subunit of the CD11b/CD18 complex found on PMNs and is identical to complement receptor CR3, the iC3B receptor). After incubation, the cells were washed, re-suspended in HBSS containing fluorescein-labeled goat anti-mouse IgG F(ab') fragment (Boehringer Mannheim Biochemicals, Indianapolis, IN), and incubated for 30 min. Subsequently, the cells were washed twice, and fixed with 500 μL of 1% formalin in phosphate-buffered saline. Cells were evaluated by means of flow cytometry to determine the quantity of CD11b expressed on the surface of the SPMNs and the percentage of cells expressing CD11b, a marker for PMNs.

Actin polymerization.—Actin polymerization of the cytoskeleton following stimulation with FMLP was quantified by means of NBD phallacidin staining (Howard and Meyer, 1984). SPMNs and BPMNs suspensions (300 μL, 3.3×10^6 cells/mL) were exposed to various concentrations of FMLP (final concentration: 10^{-9} mol/L, 10^{-7} mol/L, 10^{-5} mol/L) for one min. The reaction was stopped by fixation with 300 μL of HBSS solution containing 3.5% formalin, and 0.188 mg/mL lysophosphatidyl choline. Immediately prior to analysis by flow cytometry, cells were incubated with NBD phallacidin for 45 min (2.5 units/10^6 cells, Molecular Probes, Inc., Eugene, OR) at room temperature in the dark.

Statistical analysis.—We used Student's *t* test to test for differences between SPMNs and BPMNs. We used a paired *t* test to test for differences within the same cell population (SPMNs or BPMNs) before and after stimulation.

Results.

Isolation of SPMNs.—We collected SPMNs using the procedure described, and obtained 4-30 $\times 10^6$ cells from a 20-minute wash. Microscopically, isolated cells were mostly free of bacteria, food remnants, and epithelial cells (the proportion of epithelial cells to leukocytes was $2.3/97.7 \pm 1.7$, determined by counting six different isolations, 1000 random cells in each

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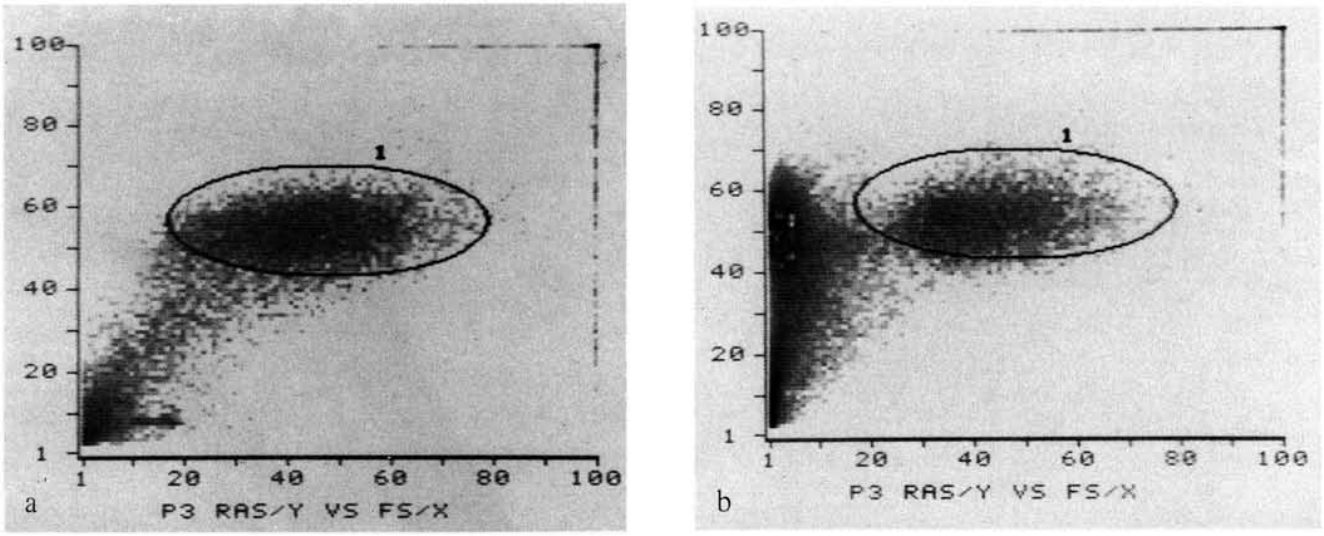


Fig. 1—Two-dimensional histogram of light scatter of SPMNs (Fig. 1a), as compared with BPMNs (Fig. 1b). Results are presented as forward light scatter on the vertical axis, and right-angle light scatter on the horizontal axis. The ellipse represents the normal range of light-scatter values for BPMNs. Flow cytometry of SPMNs (1a) shows one main population of cells, whose light-scatter values are of PMNs. Few small-size debris are seen in the left side of the Fig. Epithelial cells—whose forward light scatter is higher than that of PMNs, since they are bigger—are few. In contrast, flow cytometry of blood cells also shows lymphocytes (left of the ellipse in Fig. 1b).

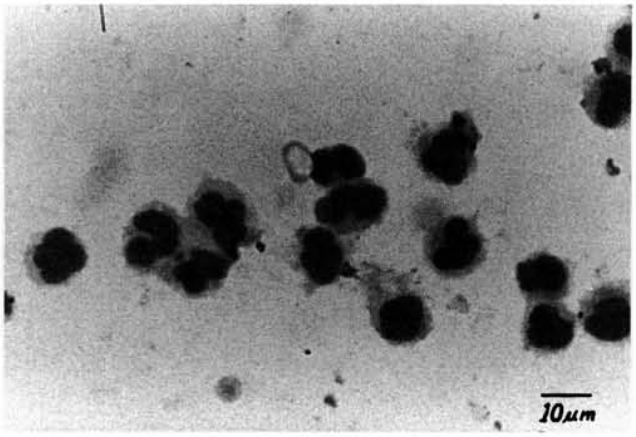


Fig. 2—Morphology of the cells obtained by the method described, as seen by light microscopy (Diff-Quik stain). Nearly all the cells are PMNs; few bacteria are seen.

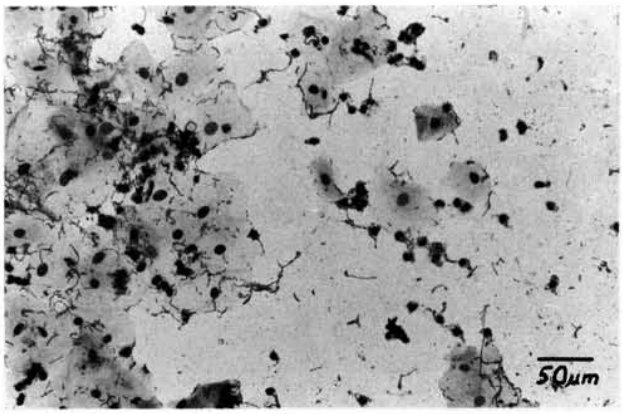


Fig. 3—A representative Fig. of the cells obtained by the previous methods described [density gradient separation, as seen by light microscopy (Diff-Quik stain)]. The sample contains many epithelial cells and bacteria, in addition to SPMNs.

case; Fig. 2). More than 99% of the leukocytes were granulocytes with from 3 to 5 lobed nuclei, while the remaining cells were mononuclear. Analysis of the isolated SPMNs by flow cytometry (Fig. 1a) showed that the pattern obtained by measuring forward *vs.* right-angle light scattering was comparable with that observed from the analysis of BPMNs (Fig. 1b). The results were confirmed by the finding that 99% of the SPMNs expressed CD11b, a marker for PMNs. The lack of cells with forward light scatter greater than SPMNs (larger cells) confirmed the microscopic observation that the sample was essentially free of epithelial cell contamination. The viability of the SPMNs was 85%. In contrast, when density gradients were used for separation (Ficoll Triosil and/or fetal calf serum, Wilton *et al.*, 1977a; Sela *et al.*, 1981) of SPMNs, the SPMNs obtained were highly contaminated with clumps of epithelial cells and bacteria (Fig. 3).

H_2O_2 production.—The level of H_2O_2 in resting SPMNs was about four times higher than that found in BPMNs ($p<0.01$; Fig. 4). This elevated level was also noted in unstimulated SPMNs held at 4°C during the incubation period. After maximal stimulation with PMA, both SPMNs and BPMNs had comparable levels of H_2O_2 ($p>0.5$). The stimulated H_2O_2 levels represented a doubling of H_2O_2 for SPMNs ($p<0.01$), while those for BPMNs increased 7.4-fold ($p<0.001$). FMLP stimulation increased H_2O_2 production in both salivary and blood PMNs by 10 to 25% in a dose-response manner ($p<0.02$). The absolute levels of H_2O_2 induced by FMLP—derived by subtracting non-stimulated H_2O_2 levels from FMLP-stimulated levels—were similar for SPMNs and BPMNs.

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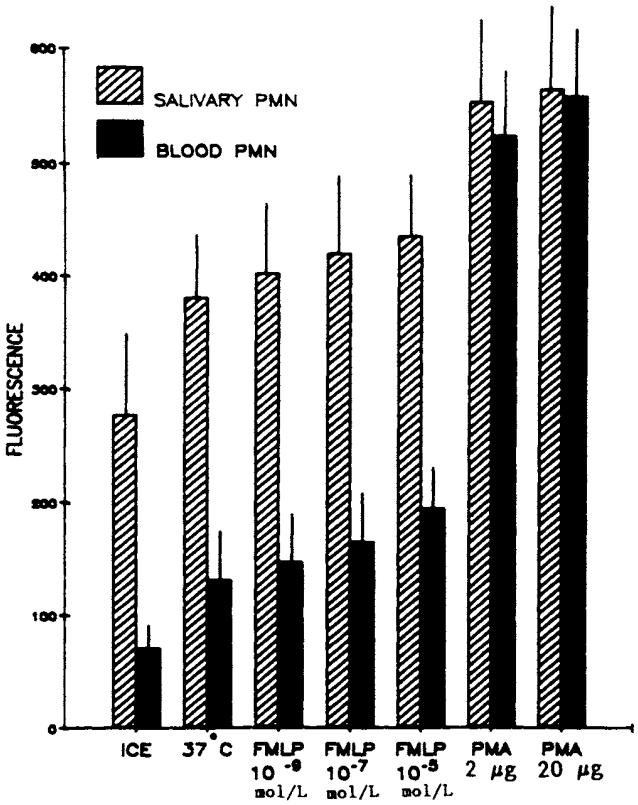


Fig. 4—H_2O_2 production by salivary and blood PMNs, at rest and after several stimuli. Results presented as the DCF fluorescence caused by the H_2O_2 production, and represent the mean ± SEM of nine determinations. SPMNs showed higher H_2O_2 production both at rest and after stimulation with FMLP. Maximal stimulation with PMA induced the same amount of H_2O_2 production in both BPMNs and SPMNs. (The concentrations of PMA represent µg/mL.)

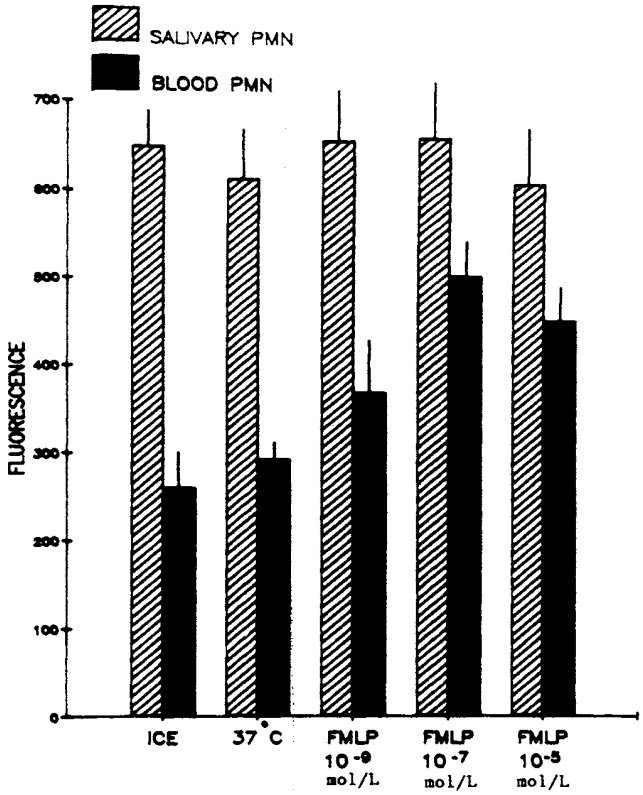


Fig. 5—CD11b expression of salivary and blood PMNs, as detected by immunofluorescence. Results represent the mean ± SEM of nine determinations. CD11b expression was higher in SPMNs than in BPMNs, both at rest and after FMLP stimulation.

CD11b expression.—CD11b expression by unstimulated SPMNs incubated on ice or at 37°C was 2 to 2.5 times higher ($p<0.001$) than that for BPMNs (Fig. 5). Stimulation of SPMNs with FMLP failed to increase ($p>0.5$) CD11b expression further, indicating that the cells were maximally upregulated. In contrast, BPMNs showed a significant increase in CD11b expression after stimulation with FMLP: Concentrations of 10^{-9} mol/L, 10^{-7} mol/L, and 10^{-5} mol/L increased CD11b expression by 41%, 93%, and 70%, respectively ($p<0.02$). Thus, SPMNs were different from BPMNs in that SPMNs maximally expressed CD11b upon collection, while BPMNs could be stimulated to upregulate CD11b. Interestingly, stimulated BPMNs expressed only 75% of the CD11b expressed by SPMNs ($p<0.05$).

F-actin polymerization.—Data on F-actin polymerization of SPMNs and BPMNs are shown in Fig. 6. The resting level of F-actin in SPMNs was twice that in BPMNs at 37°C ($p<0.01$). Stimulation of SPMNs and BPMNs with FMLP 10^{-9} mol/L, 10^{-7} mol/L, or 10^{-5} mol/L increased F-actin polymerization significantly ($p<0.01$, $p<0.001$, and $p<0.005$, respectively). Stimulation with FMLP at 10^{-9} mol/L or 10^{-7} mol/L induced comparable levels of F-actin in both cell types ($p>0.5$).

Discussion.

Two main approaches for obtaining SPMNs have been suggested previously. Klinkhamer (1963) has described a method for isolation of SPMNs for determination of biological activity and for morphological studies. He used repeated mouth washings, but noted that with his method the SPMNs were mixed with a high number of micro-organisms, food remnants, and epithelial cells as single cells, in small groups, or as sheets of cells. The proportion of SPMNs to epithelial cells was 3:1 (Klinkhamer, 1968b). The second approach made use of density gradients of fetal calf serum and/or Ficoll Triosil to separate oral leukocytes from the epithelial cells (Wilton *et al.*, 1977a; Sela *et al.*, 1981). No data have been published on the purity of the obtained SPMNs. We have attempted to use those methods, but found that SPMNs obtained were likewise contaminated, although the degree of contamination was highly variable.

In this communication, we describe a unique approach for separating salivary leukocytes from epithelial cells and oral debris based on their different sizes. Using nylon mesh, we removed clumps of epithelial cells and oral debris mechanically. Although SPMNs could conceivably adhere to the nylon mesh, we did not encounter this because of the use of a single layer of mesh, the lack of divalent cations in the collection fluid, and the large volumes used. The purity of the cells obtained was high: 97.7% of them were PMNs, and only 2.3% were epithelial cells, with 85% viability of the SPMNs.

The method described has considerable advantages over those previously used for isolating oral PMNs:

(1) It is a relatively simple procedure that does not require sophisticated laboratory equipment or reagents, or highly trained personnel, as is required to flush gingival crevices.

(2) It is relatively atraumatic to the patient, compared with flushing of the gingival crevice.

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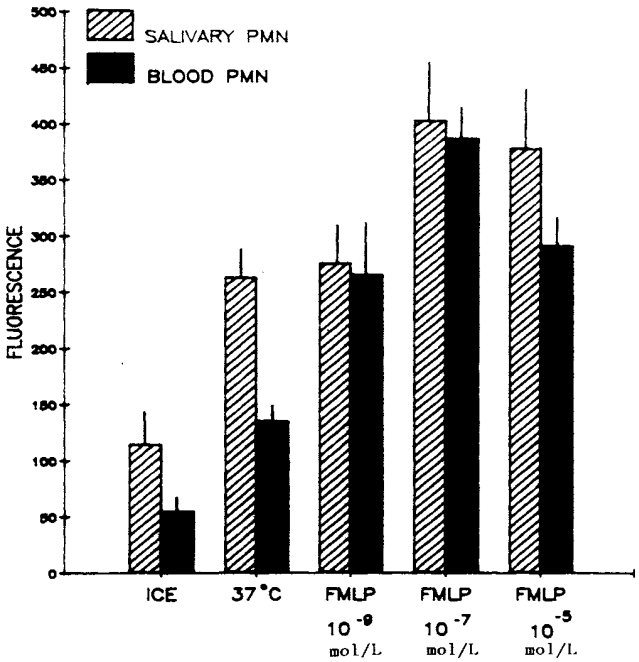


Fig. 6—F-actin polymerization of salivary and blood PMNs, quantified by NBD phallacidin fluorescence. Results represent the mean ± SEM of nine determinations. SPMNs showed higher values for F-actin polymerization than did BPMNs. At optimal stimulation (FMLP 10^{-7} mol/L), salivary and blood PMNs showed similar polymerization.

(3) It is relatively atraumatic to the SPMNs, in that they are filtered through nylon mesh just by the force of gravity, and maintained in isotonic solutions, compared with centrifugation through a density gradient.

(4) It provides SPMNs in sufficient numbers for the performance of numerous functional studies.

(5) It provides a representative sample of salivary granulocytes obtained from all sites in the mouth, as opposed to the environment of one or several gingival crevices. This is significant, since GPMNs from several pockets in the same individual can function differently, depending on the pathological condition of each pocket (Newman and Addison, 1982; Murray and Patters, 1980; Loesche *et al.*, 1988).

(6) Of all the techniques that we have examined (Klinkhamer, 1963; Wilton *et al.*, 1977a; Sela *et al.*, 1981), our method provided the highest and most consistent yield of viable SPMNs.

We observed relatively few bacteria, compared with those observed by other techniques. This is presumably because of both the large volume of washing solution used, and the fact that the bacteria adhered readily to the epithelial cells, which were efficiently removed by filtration through nylon mesh.

Our recovery of 85% viable SPMNs compares favorably with various studies that have reported a maximal viability of 56% for SPMNs and 85 to 87% for GPMNs (Scully, 1982; Sharry and Krasse, 1960; Wilton *et al.*, 1977a). The reported difference in the viability of SPMNs *vs.* GPMNs is probably related to the exposure of the cells to the detrimental factors in saliva (Klinkhamer, 1963, 1968a). We minimized the exposure of the cells to the saliva by dilution—using frequent changes of solution in the mouth—and by collecting the expectorant in a vessel containing 400 mL of HBSS-CMF at the initiation of collection. Moreover, the 10-μm pore size of nylon mesh may have removed bigger, degenerated PMNs (Raeste, 1972).

GPMNs have been comprehensively investigated (Newman and Addison, 1982; Murray and Patters, 1980; Charon *et al.*, 1982; Fanger *et al.*, 1983; Kowolik and Raeburn, 1980; Loesche *et al.*, 1988; Wilton *et al.*, 1977a,b; Scully and Wilkinson, 1985), although our knowledge on SPMNs is limited and controversies exist with regard to their functions and significance. A major factor contributing to these conflicting results has been the lack of uniformity in the techniques used to collect SPMNs. Previous studies of SPMNs have focused primarily on their morphology and the oral migratory rate; relatively few functional studies have been carried out. Functional studies of SPMNs can define whether the SPMNs' response to oral micro-organisms is ultimately protective or destructive to the oral cavity, and can clarify the role of SPMNs in preventing oral diseases.

In our study, the SPMNs were evaluated functionally by measurement of H_2O_2 production, CD11b expression, and F-actin polymerization, as compared with BPMNs. Oxidative respiratory burst is one of the main mechanisms of PMNs to kill ingested organisms. We chose H_2O_2 production to evaluate the oxidative burst development because H_2O_2 [formed by the dismutation of two molecules of superoxide (O_2^-)] is the main precursor of the oxidizing radical (OH), oxidizing halogen (HOCl), and chloramines (RNHCl). We found that SPMNs have higher resting levels of H_2O_2 ($p < 0.01$), as well as higher production of H_2O_2, after stimulation with FMLP, compared with BPMNs ($p < 0.02$). After stimulation with PMA, the production of SPMNs was comparable with that of BPMNs. This shows that the ability of SPMNs to produce H_2O_2 is not defective. The probable explanation for this difference between SPMNs and BPMNs is that the SPMNs were exposed previously to bacteria and bacterial products in the oral cavity; thus, they demonstrate high levels of H_2O_2, and even higher levels after stimulation by FMLP. Our results are in agreement with those of other studies that have shown that the intracellular bactericidal activity of GPMNs is intact (Charon *et al.*, 1982; Kowolik and Raeburn, 1980; Loesche *et al.*, 1988; and Wilton *et al.*, 1977a). However, Scully (1982) has shown decreased bactericidal activity of SPMNs compared with BPMNs and GPMNs when the cells were incubated with serum, but no difference when the cells were incubated in HBSS. The effect of serum on the functional activity of SPMNs has not been investigated.

Adhesion is a prerequisite for both phagocytosis and chemotaxis, and has been closely associated with expression of adhesion glycoproteins (Gallin, 1985; Anderson *et al.*, 1985). CD11b is the α subunit of a family of structurally and functionally related high-molecular-weight human-leukocyte surface glycoproteins. CD11b/CD18 appears to be identical to the complement receptor type 3 (CR3). The CR3 binds the inactive form (iC3b) of the third component of complement and mediates adherence and phagocytosis of iC3b-coated particles by granulocytes and monocytes. The importance of this phenomenon is demonstrated in patients with impaired adhesive ability of PMNs—when there is CD11/CD18 deficiency—who have severe periodontal disease with oral ulcerations (Waldrop *et al.*, 1987). Therefore, the importance of this molecule in the study of SPMNs cannot be overlooked. Charon *et al.* (1982) found that GPMNs have decreased adhesion to glass, and hypothesized that this might be related to a protein component in the gingival fluid (GF). Scully (1982) found reduced phagocytic activity of SPMNs, compared with blood and gingival PMNs, while Sela *et al.* (1981) found that a significantly higher percentage of SPMNs could phagocytose and bind *via* complement receptors, compared with BPMNs. Our observation of high CD11b expression in SPMNs indicates that this receptor is unaltered by exposure to the oral environment. This suggests that the reported adherence and phagocytic abnor-

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malities may be related to some external component in saliva or GF, but not to a cellular defect in CD11b expression.

The motility of the cells depends on their ability to polymerize the monomer form of actin to the polymer form (F-actin). Measuring F-actin is one way to evaluate the activity of the actin cytoskeleton polymerization system, which is an obligatory step in both phagocytosis and chemotaxis. To evaluate the reason for the reported decrease in phagocytic activity of SPMNs (Scully, 1982), we studied the ability of SPMNs to polymerize actin. SPMNs showed high F-actin content after collection, and elevated actin polymerization after stimulation with FMLP 10^{-7} mol/L ($p<0.001$). This further suggests that the decrease in phagocytic activity in the SPMNs is not caused by a defect in the actin-cytoskeleton polymerization system.

In conclusion, we have described a simple technique for isolating viable SPMNs in sufficient numbers for functional studies. We found that the collected SPMNs were stimulated as measured by H_2O_2 levels, CD11b expression, and F-actin polymerization, compared with BPMNs. However, SPMNs could significantly respond to further *in vitro* stimulus with increased H_2O_2 production and F-actin levels. Further functional studies of SPMNs will elucidate the effects of exposure to the periodontal pocket on PMN function, clarify the role of SPMNs in protecting the oral mucosa, and correlate their function with disease states.

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