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Polymorphonuclear neutrophil integrity and functionality are preserved when exposed to saliva

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ABSTRACT

Objective: Polymorphonuclear neutrophils (PMNs) are the most abundant innate immune cells and are also important effectors in the maintenance of oral health. However, little is known about the effects of saliva on the PMN. We therefore aimed to investigate the effect of saliva on the PMNs' morphology and functioning.
Design: Effect of saliva on the membrane integrity of PMNs isolated from blood was evaluated with FACS using Annexin V (apoptosis marker) and propidum iodide (membrane integrity marker). The effect on cell morphology was examined using transmission electron imaging. Binding and phagocytosis of the oral bacterium *Fusobacterium nucleatum* by PMNs was analysed by FACS. Reactive oxygen species (ROS) production was measured with chemiluminescence.
Results: Incubation with saliva for 60 min had no detectable effects on the membrane integrity or the morphology of PMNs. In contrast, preincubation of *F. nucleatum* with saliva inhibited its subsequent interaction with PMNs, resulting in a diminished production of ROS.
Conclusions: Saliva does not impair the function of PMNs. However, interaction of salivary components with *F. nucleatum* may affect their recognition by PMNs resulting in a diminished functional response.

1. Introduction

Polymorphonuclear neutrophils (PMNs) play a key role in the hosts' defence against invading pathogens (Kolaczkowska & Kubes, 2013). These innate immune cells provide the first line of defence against many pathogens, ranging from bacteria and fungi to protozoa. PMNs exert a wide range of microbicidal actions at the site of infection; these include phagocytosis, degranulation, ROS production and the formation of neutrophil extracellular traps (NETs) in order to protect the host and prevent microbial spreading (Mayadas, Cullere, & Lowell, 2014; Nicu & Loos, 2016).

The oral cavity harbours a complex microbial ecosystem (Wade, 2013), and is constantly patrolled by PMNs that migrate from the blood circulation through the oral mucosa and the gingival crevices (Attstrom & Egelberg, 1969; Delima & Van Dyke, 2003). Recruitment of PMNs towards the oral cavity involves sequential migration stages across endothelial, mucosal and epithelial barriers (Madara, 1994). Conflicting results have been obtained regarding the functional behaviour of PMNs in the healthy oral cavity. Some studies suggested that oral PMNs are fully active, kill bacteria by means of ROS production and are in a more activated condition than circulating blood neutrophils (Ashkenazi & Dennison, 1989; Landzberg, Doering, Aboodi, Tenenbaum, & Glogauer, 2015; Rijkschroeff et al., 2016; Vel, Namavar, Verweij, Pubben, & MacLaren, 1984). Other studies however, reported possible defects in oral PMNs such as a decreased response to chemotactic substances, loss of their ability to adhere or migrate, and reduced ROS production or phagocytic activity (Lukac et al., 2003; Scully & Wilkinson, 1985; Sela, McArthur, & Tsai, 1981; Wilton, Renggli, & Lehner, 1977).

It has been suggested previously that PMNs might disintegrate and

Abbreviations: CFU, colony forming units; CHWS, clarified human whole saliva; FITC, fluorescein isothiocyanate; *F. nucleatum*, *Fusobacterium nucleatum*; HRP, horseradisch peroxidase; MFI, mean fluorescence intensity; NET, neutrophil extracellular traps; PBS, phosphate buffered saline; PI, propidium iodide; PMNs, polymorphonuclear neutrophils; PS, phosphatidylserine; ROS, reactive oxygen species; RCF, relative centrifugal force; SB, saliva buffer

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rupture when coming in contact with the hypotonic salivary environment (Delima & Van Dyke, 2003). Nevertheless, the direct effect of saliva on the function of PMNs has not been investigated and we can therefore only speculate to what extent saliva may affect the properties of PMNs that enter the oral cavity. This study aimed to determine the effect of saliva on the morphology, membrane integrity and functional activity of freshly isolated peripheral PMNs that were incubated in clarified human whole saliva (CHWS). We hypothesize that saliva does not compromise the PMN morphology or its functional properties. In addition, the effect of saliva on the bacterial adhesion and phagocytosis of the oral bacterium *Fusobacterium nucleatum*, and subsequent ROS production by the PMNs was studied.

2. Materials and methods

2.1. Subjects and polymorphonuclear neutrophil isolation

Healthy volunteers were recruited from patients visiting the dental school for regular check-ups or from the non-dental personnel working at ACTA. All volunteers were informed about the purpose of this study, received written information and had given written consent prior to the start of this study. This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (World Medical, 2013). The study protocol was approved by the Medical Ethical Committee of the *VU* University Medical Centre (2012–210#B2012406). Briefly, venous blood samples from the donors were drawn into a 9 ml BD Vacutainer sodium heparin blood collection tube (BD Biosciences, Breda, the Netherlands), and maintained at room temperature. Blood samples were layered on top of 15 ml Lymphoprep™ (Axis-Shield, Oslo, Norway). After centrifugation (800*g*, 30 min, 20 °C) the red cell layer containing PMNs was harvested. Contaminating red cells were removed by hypotonic lysis (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA at pH 7.4). Subsequently, PMNs were washed and resuspended in PBS (Gibco®, Paisley, UK). After isolation, PMNs were used for the experiments within 3 h after venipuncture.

2.2. Cell counts

PMN counts were assessed using a Muse® Cell Analyzer (Merck Millipore, Darmstadt, Germany), according to the manufacturer's instruction. Samples typically contained > 90% PMNs with a cell viability ≥95% and were diluted with PBS (Gibco®) to a final concentration 1.0×10^6 cells/ml. PMNs from each volunteer were used in multiple experiments (Supplementary Table 1). Within one assay, PMNs from one donor were subjected to all conditions.

2.3. Collection of saliva and preparation of saliva buffer

Unstimulated saliva samples were collected from 6 healthy volunteers by expectoration as described previously (Prodan et al., 2015). Whole saliva was collected without stimulation, homogenized on a Vortex mixer for 1 min, and centrifuged at 10,000 RCF for 5 min to remove debris. The supernatant was pooled and transferred to a new tube, which was labelled clarified human whole saliva (CHWS). Aliquots of the pooled saliva samples were stored at −20 °C until use. In order to simulate the hypotonic ionic composition of CHWS, saliva buffer (SB) was prepared (2 mM potassium phosphate, 50 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.8) (Leung & Darvell, 1997).

2.4. Bacterial culture

F. nucleatum, strain ATCC 10953, obtained from DSMZ (Braunschweig, Germany), was cultured anaerobically (at 37 °C under 80% N_2, 10% CO_2 and 10% H_2) in a brain-heart infusion broth supplemented with 5 μg/ml hemin (Sigma-Aldrich, Schnelldorf, Germany) and 1 μg/ml menadione (Sigma-Aldrich). The bacteria were harvested from the broth cultures by centrifugation. Bacterial pellets were washed with sterile PBS (2 times), then suspended in sterile PBS to a final density of 10^9 CFU/ml, and stored at −20 °C until use.

2.5. Analysis of the cell membrane integrity

Cell membrane integrity of PMNs was analysed using the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA). Morphological changes to the cells' membrane are associated with apoptosis. Under normal conditions, phosphatidylserine (PS) is exclusively present at the cytoplasmic side of the cell membrane. This method is based on the binding of FITC-Annexin V to PS molecules, which in apoptotic cells become exposed on the outer leaflet of the cell membrane. In addition, membrane integrity was evaluated using propidium iodide (PI), a membrane impermeant DNA-binding probe. Viable cells with fully intact membranes will therefore stain negative for both Annexin V and PI (van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998; Vermes, Haanen, Steffens-Nakken, & Reutelingsperger, 1995). In order to evaluate the effect of saliva on the cells' morphology, PMNs were added to CHWS, SB or PBS at room temperature, and the proportion of viable PMNs, e.g. non-apoptotic cells, was analysed. After 60 min incubation, all samples were centrifuged (500 RCF, 10 min, 4 °C), and the pellet was resuspended in 50 μl PBS. The cell suspension was subsequently incubated for 15 min in the dark at room temperature with 2.5 μl FITC-Annexin V and 2.5 μl PI, prior to fixation with 200 μl Annexin V binding buffer (0.01 M HEPES/NaOH pH 7.4, 0.14 M NaCl, 0.0025 M $CaCl_2$). Flow cytometric analysis was performed within one hour on a BD Accuri™ C6 flow cytometer (BD Biosciences). Cells staining negative for both Annexin V and PI were considered viable cells and were expressed as the percentage viable PMNs from the total PMN suspension.

2.6. TEM imaging

Transmission electron microscopy (TEM) was used to evaluate the effect of CHWS on the cell morphology. PMNs were directly fixated with McDowells fixative (1% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer), or first added to CHWS, SB, or PBS for 60 min at room temperature, and then centrifuged (500 RCF, 10 min, 4 °C) prior to fixation. Samples were washed with PBS and were post-fixed with a solution of 1% osmium tetroxide (OsO_4) in water. Subsequently, the specimens were dehydrated in an ethanol series and embedded in epoxy resin (LX112). For electron microscopic analysis, ultrathin (80 nm) sections of the samples were cut with a diamond knife, collected on formvar-coated grids (Sigma-Aldrich) and counterstained with uranyl acetate and lead citrate. All samples were sectioned, examined and photographed in a FEI Tecnai T12 transmission electron microscope. Cell circumference, nuclei circumference and ratios of heterochromatin versus euchromatin were determined using Image-Pro Plus 7.0.

2.7. Bacterial adhesion and phagocytosis analysis

Bacterial adhesion and phagocytosis of *F. nucleatum* by PMNs were analysed using flow cytometry. *F. nucleatum* (10^9 CFU/ml) was labelled with 100 μg/ml FITC (Invitrogen, Landsmeer, The Netherlands) in carbonate buffer (pH 9.5) for 30 min at room temperature. To remove free FITC, *F. nucleatum* were washed three times with PBS and resuspended in RPMI, supplemented with 20 mM HEPES (Gibco®) to a concentration of 5×10^7 CFU/ml and stored in aliquots at −80 °C until use.

For the measurement of bacterial adhesion and phagocytosis, PMNs were incubated aerobically with CHWS, SB or PBS for 30 min at 0 °C and 37 °C, respectively. Samples contained 50 μl PMNs (2.5×10^5 in RPMI, supplemented with 20 mM HEPES), 50 μl FITC-labelled *F.*