

## Chemotactic Activity of Human Neutrophils to *Streptococcus mutans*

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### Abstract

**Objective:** The aim of this study was to evaluate chemotactic activity of neutrophil to *S. mutans*. Chemotaxis assay was performed in blind well chambers. **Materials & Methods:** Hanks balanced salt solution (HBSS) containing  $10^6$  *S. mutans*,  $10^8$  *S. mutans*,  $10^{-8}$  M fMLP, or HBSS alone were placed in the lower wells of the chamber and covered with polycarbonate membrane filter. Neutrophils suspension ( $2 \times 10^5$  cells) was then placed in the upper compartment. After incubation for 60 mins at  $37^\circ\text{C}$  in a humidified atmosphere with 5%  $\text{CO}_2$ , the filters were removed and stained with Giemsa. **Result:** ANOVA revealed statistically significant differences among groups ( $p < 0.05$ ), indicating that *S. mutans* induced neutrophils chemotaxis. The number of neutrophils migration in response to  $10^8$  *S. mutans* and  $10^6$  *S. mutans* were significantly greater compared to fMLP ( $p < 0.05$ ). **Conclusion:** *S. mutans* may activate human neutrophils, resulting in the chemotaxis of the neutrophils.

**Keywords:** chemotaxis, neutrophils, *Streptococcus mutans*

### Introduction

*Streptococcus mutans* has been most frequently isolated from human dental plaque and implicated as the main etiological agent of dental caries.<sup>1,2</sup> Previous studies revealed that *S. mutans* was detected from deep carious lesions and pulp necrotic<sup>3</sup>. This bacteria may invade the pulp tissue

through the exposed dentinal tubules and thus, invokes immune response in the pulp.<sup>4,5</sup>

Neutrophils are part of the innate immune defense system and the first line of protection against bacterial infection. Neutrophils are recruited to the site of infection by sensing and migrating toward a gradient of chemotactic substance.

These cells then kill invading bacteria by phagocytosis.<sup>6</sup>

Chemotaxis of neutrophils is critical to establish an acute inflammatory response which may cause tissue injury as seen in a variety of inflammatory disorders. Accordingly, the pathways by which signals from chemoattractants induce neutrophils response are of considerable importance to the mechanism of the diseases.<sup>7</sup>

*In vitro* studies have shown that chemotaxis of neutrophils may be a direct effect of bacteria and that the ability to elicit neutrophils chemotaxis may differ among different oral microorganisms. Previous studies revealed that *Actinomyces viscosus*, *Treponema denticola* and *Bacteroides melaninogenicus* have a potential to induce neutrophils chemotaxis.<sup>8</sup> However, it remains unknown whether *S. mutans* have ability to promote neutrophil chemotaxis. The purpose of this study was to evaluate chemotactic activity of neutrophil to *S. mutans*.

## Material and methods

### *S. mutans* preparation

*S. mutans* (ATCC 10449) was maintained by weekly subculture on brain heart infusion (Difco Laboratories, Detroit, MI) agar plates

supplemented with 5% sheep blood, 1% yeast extract (Difco Laboratories, Detroit, MI) and hemin. *S. mutans* were grown in an anaerobic chamber at 37°C for 72 hours. The cells were then scraped and aseptically suspended in Hanks balanced salt solution (HBSS). After washing 3 times, the cells were dispersed by vortexing and repeated passages through a pipettor tip. Cells were adjusted to an optical density of 0.3 at 560 nm to give 10<sup>8</sup> cells/ml in HBSS.

### Preparation of N-formyl-L-methionyl-L-leucyl-L-phenylalanine

One mg of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Sigma, USA) was dissolved in 2285.4 µl of dimethylsulfoxide and diluted with 2057 µl HBSS, which gave a concentration of 10<sup>-3</sup>M and stored at -20°C before use. The stock solution was further diluted to concentration of 10<sup>-8</sup>M with HBSS, and used as a positive control for the chemotaxis assay.<sup>9,10</sup>

### Neutrophils isolation

Heparinized blood was drawn from a healthy volunteer with informed consent. The neutrophils were isolated by Ficoll-Hypaque density centrifugation followed by monopoly resolving

medium (Dainippon Sumitomo Pharma Co. Ltd, Japan) density centrifugation. Erythrocytes contamination in the neutrophil fraction was removed by hypotonic lysis with sterilized distilled water. After washing three times with phosphate buffered saline, the cells were suspended in HBSS. More than 99% of the isolated neutrophils were viable, as judge by the trypan blue dye exclusion method. The purity of the neutrophils used in this study was more than 98%.

**Chemotaxis assay**

The *in vitro* chemotaxis assay was performed in blind well chambers (Neuro Probe, Inc., Gaithersburg, USA). HBSS 100  $\mu$ m containing  $10^6$  *S. mutans*,  $10^8$  *S. mutans*,  $10^{-8}$  M fMLP or medium (HBSS) alone were placed in the lower wells of the chamber. The lower wells were covered with 3  $\mu$ m pore size polycarbonate membrane filter (Neuro Probe Inc., Gaithersburg, USA), and then 200  $\mu$ m neutrophils suspension ( $2 \times 10^5$  cells) was placed in the upper compartment. After incubation for 60 mins at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, the filters were removed. Non-adherent cells from the upper side of the filter were removed by

drawing the filter across a cotton bud and rinsing in HBSS. The filters were then fixed in 100% methanol and stained with Giemsa.

The chemotactic activity was evaluated microscopically by taking the average counts in 3 random highpower (x 400) fields of the number of neutrophils found at the bottom side of the membrane. Results of chemotactic activity were expressed as the difference between the number of cells migrating towards *S. mutans* or fMLP minus the number of cells migrating towards HBSS.

**Statistical analysis**

Each experiment was done in triplicate. ANOVA followed by LSD was used to detect statistically significant differences among experimental groups. A p<0.05 was considered statistically significant.

**Table 1.** Neutrophils migration after stimulation

Group	X $\pm$ SD
$10^8$ <i>S. mutans</i>	102.67 $\pm$ 5.13
$10^6$ <i>S. mutans</i>	95.67 $\pm$ 1.53
fMLP	68.67 $\pm$ 8.08

## Results

The data demonstrated an increasing number of neutrophils migration toward  $10^8$  *S. mutans* and  $10^6$  *S. mutans* in comparison with fMLP (Table 1). ANOVA revealed statistically significant differences among groups ( $p < 0.05$ ), indicating that *S. mutans* induced neutrophils chemotaxis.

The number of neutrophils migration in response to  $10^8$  *S. mutans* and  $10^6$  *S. mutans* were significantly greater compared to fMLP ( $p < 0.05$ ). No statistically significant difference was found between the number of neutrophils migration toward  $10^8$  *S. mutans* and  $10^6$  *S. mutans* ( $p > 0.05$ ).

## Discussion

The present study demonstrated that *S. mutans* induced neutrophils migration, indicating that *S. mutans* have ability to promote chemotaxis of human neutrophils.

Neutrophils chemotaxis is a complex process which requires integrated pathways including actin polymerization, cytoskeletal reorganization, morphological polarization, specific adhesiveness, and cell-substratum detachment. An early event following exposure of the cell to a chemoattractant is a shape change

with transformation to a torpedo-like morphology. The cell is aligned in its long axis in the direction of the gradient, with polarization of the receptors for the chemoattractant at the leading edge. Movement of the cell through the gradient towards a region of greater concentration of the chemoattractant requires selective adhesion of the cell at its leading edge to the extracellular matrix adjacent cells, and alignment of the microfilamentous elements of the cytoskeleton in the direction of the gradient. Thereafter, there is contraction of the microfilaments with simultaneous release at the trailing edge of the cell. In this way the body of the cell is pulled up through the gradient.<sup>11</sup>

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) may induce chemotaxis by binding with specific G-protein coupled receptors expressed on neutrophils. The interaction of fMLP with its receptor triggers multiple second messengers, through the activation of phospholipase (PL) C, PLD and PLA2, and rapidly stimulates phosphatidylinositol-3-kinase, as well as activating tyrosine phosphorylation. This pathway also induces an increase in intracellular level of cAMP and the involvement of protein kinase C and mitogen

activated protein kinases which are strongly associated with the chemotactic activity of the neutrophils.<sup>7,10</sup>

*S. mutans* was found in deep carious lesions and may initiate pulpal infection. This bacteria may invade the pulp tissue through the exposed dentinal tubules and therefore, elicit inflammatory response in the pulp.<sup>3,12</sup> During inflammatory response associated with bacterial infections, neutrophils migrate into tissue containing viable bacteria and participate in their elimination from the tissue by phagocytosis. During phagocytosis, the neutrophils produce lysosomal enzymes which act as microbicidal substances on the ingested microorganisms.<sup>13,14</sup> However, in severe reactions, the same enzymes may be liberated into the extracellular space and injure the pulp tissue leading to pulpal necrosis and development of periapical lesions.<sup>15,16</sup>

In conclusion, the present study demonstrated that *S. mutans* activated human neutrophils resulting in the chemotaxis of the neutrophils. Thus, the bacteria may participate in the pathogenicity of the pulpal diseases. However, further works are required to identify any specific chemotactic components of *S. mutans* and exact mechanism by which

neutrophils are attracted in response to *S. mutans*.

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