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HUMAN NEUTROPHIL CHEMOTACTIC RESPONSE TO ACTINOMYCES ISRAEL II

(RESPONS KHEMOTAKSIS NETROFIL TERHADAP ACTINOMYCES ISRAEL II)

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Abstract

Apical periodontitis is an inflammation and destruction of periradicular tissues caused by bacteria of endodontic origin. *Actinomyces israel II* has been consistently isolated from the periapical tissue of teeth which did not respond to proper conventional endodontic treatment. Inflammatory processes are characterized by the dynamic influx of neutrophils which is initiated by chemical signals including chemotactic factors. The purpose of this preliminary study was to evaluate chemotactic response of neutrophil to *A. israel II*. Chemotactic activity was performed in vitro with blind well chambers. Hanks balanced salt solution (HBSS) containing 10^4, 10^6, and10^8 *A. israel II*, 10^{-8} M N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), or HBSS were placed in the lower wells of the chamber and covered with 5 µm polycarbonate membrane filters. Neutrophils suspension ($2X10^5$ cells) was placed in the upper compartment and incubated for 60 mins at 37°C in a humidified atmosphere with 5% CO_2. The filters were then removed and stained with Giemsa. Statistical analysis used ANOVA test. The result showed that there were significant differences of the number of neutrophils among groups ($p<0.05$), indicating that *A. israel II* induced neutrophils chemotaxis. The number of neutrophils migrations in response to 10^6, and10^8 *A. israel II* were significantly greater compared to 10^{-8} M fMLP ($p<0.05$). In conclusion, *A. israel II* directly induced chemotactic response of neutrophil, therefore, *A. israel II* may contribute to the pathogenesis of apical periodontitis through modulation of the host innate immune response.

Key words: chemotactic response, neutrophil, *Actinomyces israel II*

Abstrak

Periodontitis apikalis merupakan suatu penyakit inflamasi dan destruksi jaringan periradikuler yang disebabkan oleh bakteri-bakteri endodonti. *Actinomyces israel II* merupakan bakteri yang dapat diisolasi dari jaringan periapikal gigi yang mengalami kegagalan perawatan endodonti konvensional. Pada proses inflamasi terjadi migrasi netrofil menuju tempat yang terinfeksi yang diinisiasi oleh sinyal-sinyal kimiawi termasuk faktor-faktor khemotaksis. Tujuan penelitian pendahuluan ini adalah untuk mengetahui respons khemotaksis netrofil terhadap bakteri *A. israel II.* Penelitian aktivitas khemotaktik ini dilakukan secara *in vitro* dengan menggunakan *blind well chambers.* Bakteri *A. israel II* konsentrasi 10^4, 10^6, dan10^8 serta 10^{-8} M N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) dalam *Hanks balanced salt solution* (HBSS), atau HBSS ditempatkan dalam sumuran bagian bawah dari *blind well chambers,* dan ditutupi dengan filter membran *polycarbonate* dengan pori-pori berdiameter 5 µm. Suspensi netrofil ($2X10^5$ sel) ditempatkan pada sumuran bagian atas. *Blind well chambers* selanjutnya diinkubasi selama 60 menit pada suhu 37°C dengan 5% CO_2. Filter selanjutnya diambil dan diwarnai dengan Giemsa. Uji statistik menggunakan test ANOVA. Hasil penelitian menunjukkan perbedaan jumlah netrofil antar kelompok ($p<0.05$), mengindikasikan bahwa *A. israel II* menginduksi khemotaksis netrofil. Jumlah netrofil yang bermigrasi sebagai respon terhadap *A. israel II* konsentrasi 10^6 dan10^8 lebih besar secara signifikan bila dibandingkan dengan 10^{-8} M fMLP ($p<0.05$). Kesimpulan penelitian ini adalah bakteri *A. israel II* secara langsung mampu menginduksi respons khemotaktik netrofil, dengan demikian bakteri *A. israel II* berperan terhadap patogenesis dari periodontitis apikalis melalui modulasi respons imun inang.

Kata kunci: respons khemotaksis, netrofil, *Actinomyces israel II*

INTRODUCTION

Apical periodontitis is an inflammation of periodontal tissue in the area around the root apex of the tooth. This disease is a sequel to endodontic infection. Initially, the tooth pulp becomes infected and necrotic by an autogenous oral microflora. The microbial invaders in the root canal can advance, or their products can enter into the periapex.[1] In response, the host defenses consisting of several classes of cells, intercellular messengers, anti-bodies, and effector molecules. The dynamic encounter between microbial factors and host defenses at the interface between infected radicular pulp and periodontal ligament results in the formation of various periapical lesions.[2]

Actinomyces israelii is an anaerobic Gram-positive pleomorphic rod bacteria. This bacteria is primarily involved in extraradicular infection and has been repeatedly isolated in the periapical tissues of cases which do not respond to conventional endodontic treatment.[3] Immunohistochemical markers have provided good evidence for the presence of *A. israelii* in confirmed well-organized but treatment-resistant periapical tissue lesions.[4]

In the cases of apical periodontitis, a dense infiltration of immunocompetent cells is seen in periradicular lesions and may induce bone resorption.[1] Neutrophils are part of the innate immune defense system and the first line of protection against bacterial infection.[5] Neutrophils are recruited to the site of infection by sensing and migrating towards a gradient of chemotactic substance. These cells then kill invading bacteria by phagocytosis.[6]

Neutrophils might be attracted to inflammatory sites by chemotaxis, ie, a unidirectional migration of cells through a concentration gradient towards the source of a soluble chemoattractant molecule.[7,8] Chemotaxis of neutrophils plays an important role in inducing an acute inflammatory response which may cause tissue injury. Therefore, the pathways by which signals from chemoattractants stimulate the response of neutrophils are considerable importance to the mechanism of the diseases.[9] *In vitro* studies have shown that chemotaxis of neutrophils may be a direct effect of bacteria.[10,11] Until now, the precise pathological mechanism of apical periodontitis remains unclear. This preliminary research aimed to study the involvement of *A. israelii* in the pathogenesis of apical periodontitis by evaluating the chemotactic response of neutrophil to *A. israelii*.

MATERIALS AND METHODS

This study was an experimental laboratory study, which was done in Laboratory of Microbiology, Niigata University, Japan. *A. israelii* (ATCC 10048) 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. Inoculated plates were incubated in an anaerobic chamber at 37°C for 7 days. The bacteria harvested, washed and suspended in Hanks balanced salt solution (HBSS). Cells were then adjusted to an optical density of 0.1 at 625 nm to get 10^8 cells/ml in HBSS.

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Sigma, USA) was prepared at 10^{-3}M in dimethyl sulfoxide and stored at -20°C before using. The stock solution was further diluted with HBSS in concentration with of 10^{-8}M, and used as a positive control for the chemotaxis assay.

The neutrophils were isolated from human heparinnized blood by Ficoll-Hypaque density gradient centrifugation for 20 min at 1600 rpm followed by monopoly resolving medium (Dainippon Sumitomo Pharma Co. Ltd, Japan) density gradient centrifugation for 30 min at 1600 rpm. The venous blood was obtained from a healthy volunteer with informed consent. Contaminating erythrocytes were removed by hypotoniclysis with sterilized distilled water. The isolated cells were then washed and resuspended in HBSS to a concentration of 5-6X10^6 cells/ml. The viability of the cells was determined by trypan blue exclusion. The cells were used if the viability and purity were more than 98%.

All assays were performed in triplicate using blind well chambers (Neuro Probe, Inc., Gaithersburg, USA) which were separated by 5 µm pore size polycarbonate membrane filter (Neuro Probe Inc., Gaithersburg, USA). The lower part of the chamber was filled with 100 m HBSS containing 10^4, 10^6, 10^8 *A. israelii*, 10^{-8} M fMLP or medium (HBSS) alone. The upper compartment was filled with 200 µm neutrophils suspension (2X10^5 cells). The chambers were incubated for 60 mins at 37°C in a humidified atmosphere with 5% CO_2. The filters then 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 stained cells found at the bottom side of the membrane were observed microscopically (X400 magnification). The chemotactic activity was evaluated by taking the average counts in 3 random fields. Data was expressed as the number of cells migrating towards *A. israelii* or fMLP minus the number of cells migrating towards HBSS.

Statistical analysis used ANOVA test, followed by least significance difference (LSD) test. A $p<0.05$ was considered statistically significant.

RESULTS

Data showed an increasing number of neutrophils migration in a concentration of bacteria dependent manner (Table 1; Figure 1). ANOVA revealed statistically significant differences of the number of neutrophil among groups ($p<0.05$), indicating that *A. israelii* induced neutrophils chemotaxis. The number of neutrophils migration in response to 10^6 and 10^8 *A. israelii* were significantly higher compared to fMLP ($p<0.05$). There was no significant difference between the number of neutrophils migration towards 10^6 *A. israelii* and 10^8 *A. israelii* ($p>0.05$).

Table 1. Neutrophils migration after stimulation

Group	X±SD
10^2 *A. israelii*	166±26
10^4 *A. israelii*	207±17
10^6 *A. israelii*	279±17
10^8 *A. israelii*	280±23
fMLP	245±10

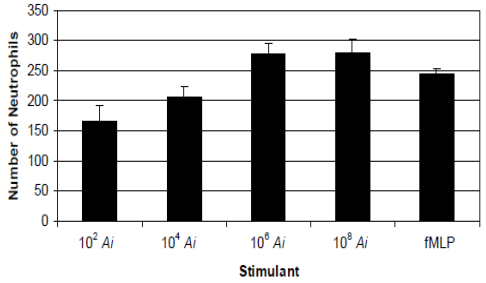

Figure 1. Neutrophils migration after stimulation

DISCUSSION

The present study revealed that *A. israelii* induced neutrophils migration, indicating that *A. israelii* has an ability to promote chemotaxis of human neutrophils. A primary event in the inflammatory response is accumulation of neutrophils at the site of inflammation. 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.[6] However, in severe reactions, the same enzymes may be liberated into the extracellular space and injure the tissue leading to necrosis and development of periapical lesions.[2] Therefore, coordinated activation and recruitment of neutrophils through chemotaxis to the infected area are essential and are the crucial step in the inflamatory response.[12]

All assays in this study were performed using modified blind well chamber. This method uses a two-chambered compartment divided by a micropore filter. Neutrophils are placed in the top compartment of the chamber and the material to be tested for chemotaxis activity is placed in the lower compartment. Cells migrate from the upper to the lower surface of the filter with the number of migrating cells dependent on the chemotaxis potency of the material in the lower compartment. Migration is measured by counting the neutrophils that had moved through the filter pores to the underside of the membrane in response to presentation of the chemoattractant. This method provided a rapid, quantitative measure of the chemoattractant capacity of a given substance.[13]

Agents associated with chemotaxis activity can be classified as those that react directly with the neutrophils and influence its migration, or those that must activate host serum factors to generate neutrophils chemotaxis activity. Bacteria and their components and products have been known to exert a direct chemotaxis influence on neutrophils.[10,11]

Our study showed that *A. israelii* induced neutrophils chemotaxis activity. However, this study did not evaluate active chemotactic component of the bacteria. This study proved that *A. israelii* has a direct chemotactic factor for human neutrophils. Therefore, the bacteria may participate in the pathogenicity of the periodontitis apicalis indirectly by eliciting neutrophil activation as well as directly by the virulence of its components and products.

N-formyl-L-methionyl-L-leucyl- L- phenylalanine (fMLP) was used as a positive control in this study. This substance may induce chemotaxis by binding with specific G-protein coupled receptors expressed on neutrophils. The binding subsequently triggers multiple second messengers, through the activation of phospholipase (PL) C, PLD and PLA2, and rapidly stimulates phophatidylinositol-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.[14,15]

In conclusion, *A. israelii* directly induced chemotactic response of neutrophils; therefore, *A. israelii* may contribute to the pathogenesis of apical periodontitis through modulation of the host innate

immune response. However, identification of any specific chemotactic components of *A. israelii* and mechanism by which neutrophils were attracted in response to *A. israelii* require further examination.

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