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Enhanced phagocytosis of *Aggregatibacter actinomycetemcomitans* cells by macrophages activated by a probiotic *Lactobacillus* strain

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ABSTRACT

The activation of phagocytosis is one important approach to clearing pathogenic cells in a host. This study evaluated the ability of probiotic lactobacilli to induce phagocytic activity as well as the clearance of a periodontal pathogen, *Aggregatibacter actinomycetemcomitans*. First, the activation of phagocytosis was found by using lyophilized dead cells. Probiotic *Lactobacillus* strains significantly enhanced the phagocytic activity of macrophage cells, indicating that the probiotic lactobacilli have a remarkable ability to stimulate the macrophages. Essentially, 3 *Lactobacillus* strains tested did not have any critical toxic effect on the murine macrophage, and *Lactobacillus johnsonii* NBRC 13952 showed the least cytotoxic effect on the RAW264.7 macrophages. The expression of classically activated macrophage markers, IL-1β, and cluster of differentiation 80 increased by *L. johnsonii* NBRC 13952; however, there was no significant difference for IL-18. The highest phagocytic activity by macrophages was found in a condition in which the macrophage activated by *L. johnsonii* NBRC 13952 functions to kill the cells of *A. actinomycetemcomitans*. Correlating with the result, a high amount of hypodiploid DNA (SubG1) was detected from the macrophage cells stimulated by *L. johnsonii* NBRC 13952. Taken together, the results suggest that macrophages activated by the *Lactobacillus* strain can facilitate the phagocytosis of *A. actinomycetemcomitans* cells by linking with enhanced apoptotic activities. In conclusion, *L. johnsonii* NBRC 13952 has a certain role in activating the RAW264.7 macrophages, thereby counteracting the infection of *A. actinomycetemcomitans*.

Key words: phagocytosis, probiotic, periodontal pathogen, macrophage activation, apoptosis

INTRODUCTION

Macrophages are key phagocytes in the innate immune response against invading microorganisms and play a significant role in the modulation of an inflammatory environment in the tissues (DeLeo, 2004). The response is influenced by the local microenvironmental signals (Murray and Wynn, 2011). In general the initial responder, macrophages usually trigger an inflammatory response and produce proinflammatory mediators such as tumor necrosis factor (**TNF**), IL-1, IL-6, IL-18, and IL-12 (Cohen, 2002). The response promotes the production of various antimicrobial factors and nitrogen intermediates that are highly toxic to kill infecting microorganisms (Murray and Wynn, 2011). However, excessive or prolonged macrophage activity for the bacterial clearance may trigger damage of the neighboring tissues at the infected site (Benoit et al., 2007). Finally, normal cell tissues are damaged through excessive responses by immune cells (Silva et al., 2015). Pathogens such as *Aggregatibacter actinomycetemcomitans* might be able to alter the modulation of leukocytic apoptosis or the cell death of macrophages by which the host immune response may be adversely influenced (Meyer et al., 1996; DeLeo, 2004). Basically, a normal treating process for the bacterial infection is the phagocytic activity and neutrophil apoptosis to kill the infecting bacteria, and then the apoptotic neutrophil cells are removed by macrophages (DeLeo, 2004). However, the normal pathway might be altered by the pathogens, which can use several survival strategies such as escaping the phagosome, inducing the lysis of neutrophil cells, and delaying the process of apoptosis to promote the propagation of intracellular pathogens (Meyer et al., 1996; DeLeo, 2004). A previous study reported the survival of internalized *A. actinomycetemcomitans* cells inside the murine macrophage in the infection model by confocal scanning microscopy (Kato et al., 1995). This activity halts the cell cycle at the G1 phase through the suppression of p21 expressed and the degradation of cy-

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clin D1, resulting in the induction of apoptotic cell death (Kasai et al., 2010; Okinaga et al., 2013). In addition, the biofilm of *A. actinomycetemcomitans* increased in a porous-type hydroxyapatite (**HA**) rather than a dense-type HA, although biofilm dispersal was observed with time (Jaffar et al., 2016b). The property of porous-type HA is similar to dentin, and the property of dense-type HA is similar to enamel in the tooth (Sakaguchi and Powers, 2012). The biofilm formed at the dentinal tubule may cause the invasion of pathogenic cells through the root of the dentinal tubule or pulp cavity (Li et al., 2000). Therefore, an alternative approach via the use of probiotic bacteria may be promising to combat the pathogenic strain, as the mature biofilm of *A. actinomycetemcomitans* cells was degraded by probiotic bacteria (Jaffar et al., 2016a). The approach has several advantages due to the effect of probiotic strains, which are able to modulate the immune response (Oelschlaeger, 2010; Jang et al., 2013, 2014; Ji et al., 2013). The macrophage cells might be activated by lactobacilli, which stimulate the production of both inflammatory and anti-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-10, and TNF-α (Morita et al., 2002; Ji et al., 2013). However, to the best of our knowledge, there are so far no reports on the relationships between immune response, probiotic bacteria, and pathogenic bacteria. In this study we evaluated phagocytic activity by macrophage cells using the murine macrophage cell line RAW264.7 as an immune cell, lactobacilli as probiotic bacteria, and *A. actinomycetemcomitans* as a pathogen. In particular, we determined the efficiency of lactobacilli-activated macrophages in the presence or absence of *A. actinomycetemcomitans*.

MATERIALS AND METHODS

Cell Culture

The murine macrophage cell line RAW264.7 was grown in α-minimum essential medium (**α-MEM**; Gibco Laboratories, Grand Island, NY) supplemented with 5% fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 μg/mL) at 37°C inside a 5%-controlled CO_2 incubator.

Bacterial Strains and Culture Conditions

Aggregatibacter actinomycetemcomitans strain Y4 was obtained from Kyushu Dental University, Japan, and routinely grown in brain–heart infusion medium (Difco Laboratories, Detroit, MI) supplemented with 1% yeast extract (wt/vol) by shaking at 120 rpm at 37°C and 5% CO_2 for 48 h. In addition, probiotic bacteria *Lactobacillus johnsonii* NBRC 13952, *Lactobacillus plantarum* NBRC 15891, and *Lactobacillus fermentum* NBRC 15885 were obtained from Kitakyushu College of Technology, Japan. All probiotic bacteria were grown in De Man, Rogosa and Sharpe medium supplemented with l mL/L of Tween 80 (Fluka, Sigma-Aldrich, St. Louis, MO) under anaerobic conditions at 37°C for 48 h.

Preparation of Lyophilized Dead Bacterial Cells

Overnight cultures of *A. actinomycetemcomitans* Y4 and probiotic lactobacilli were centrifuged at 12,063 × *g* for 10 min. Each cell pellet was collected and washed twice using autoclaved distilled water, and the pellet suspension was adjusted to a cell concentration of 2×10^{10} cells/mL and autoclaved at 120°C for 15 min. Last, all samples were lyophilized using a freeze dryer (Labconco, Kansas City, MO), and the powder forms were preserved at −20°C for further experiments.

Screening of Phagocytosis Activity

The phagocytic activity of macrophages was determined using a phagocytosis assay kit (Cayman Chemical Co., Ann Arbor, MI). The RAW264.7 cells were cultured in tissue-culture sets (1×10^6 cell/mL), seeded in 6-well plates (Corning Inc., Corning, NY) at a concentration of 4×10^5 cell/mL, and incubated at 37°C and 5% CO_2. Each pellet suspension (2 mL) of dead cells of *A. actinomycetemcomitans* Y4 or probiotic bacteria, *L. johnsonii* NBRC 13952, *L. plantarum* NBRC 15891, and *L. fermentum* NBRC 15885 was mixed with the cell suspension of RAW264.7 macrophages to make a mixture at the multiplicity of infection (**MOI**) of 1,000:1 (bacterial cells:macrophage cells) in α-MEM. A sample without any bacterial suspension was used as a negative control. All the samples were incubated for 24 h at 37°C and 5% CO_2. The determination of phagocytic activity was performed using an assay reagent, latex bead–rabbit IgG–fluorescein isothiocyanate (**FITC**; Cayman Chemical Co.). Initially, the latex beads were diluted 40 times using α-MEM, and the diluted reagent (with beads) was replaced with the supernatant of the above mixture (after 24 h) and incubated at 37°C for 2 h in the 5% CO_2 incubator. The IgG molecules bind with a protective protein and fragment crystallizable receptor on the surface of the activated macrophage cells; thereby, the uptake of the beads was facilitated. Thus, the presence of beads inside the macrophage cells was determined as the phagocytic activity by detecting the fluorescence at 498 nm of the excitation wavelength and at 522 nm of the fluorescence wavelength. The number of activated macrophage cells with the beads was counted using the All-in-One fluorescence micro-