Effects of Enterococcus faecalis sonicated extracts on IL-2, IL-4 and TGF-β1 production from human lymphocytes

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ABSTRACT

In order to examine the immunoresponse of host cells to Enterococcus faecalis, this in vitro study monitored the production of Interleukin-2 (IL-2), Interleukin-4 (IL-4) and Transforming growth factor-β1 (TGF-β1) in human lymphocytes. Lymphocytes were activated with PHA in the presence or absence of sonicated extracts of E. Faecalis (SEF) and further incubated for 72 hours. The level of each cytokine was measured by ELISA. Data were analyzed with Kruskal-Wallis test and Mann-Whitney U test (P < 0.05). PHA-activated group did exhibit higher level of IL-2 and IL-4 than untreated control group. The levels of expression of both cytokines were significantly decreased following the treatment of high (25 ㎍/㎖) and medium concentration (12.5 ㎍/㎖) of SEF (P < 0.05) than those of PHA activated group. But low concentration (5 ㎍/㎖) of SEF showed the similar level of IL-2 and IL-4 production as those of PHA activated group. TGF-β1 was unaffected by SEF treatment. These results suggested that E. faecalis may suppress IL-2 and IL-4 production by lymphocytes and this could be one of possible factors why E. faecalis are found frequently in the teeth with failed endodontic treatment. [J Kor Acad Cons Dent 30(1):1-6, 2005]

Key words: Enterococcus faecalis, Lymphocyte, IL-2, IL-4, TGF-β1, Sonicated extract

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whereas the other organisms died within 4 to 48 hr. E. faecalis maintained the capability to invade dentinal tubules and adhered to collagen in the presence of human serum. Survival of E. faecalis in calcium hydroxide at high pH was related to a functional proton pump.

However, there have been few investigations about the immunologic effect of E. faecalis. In previous studies, sonic extracts from bacteria were studied for their effects on the immune response. For example, sonicated material of Fusobacterium nucleatum can evoke a concentration-dependent stimulatory or suppressive effect on the proliferation rate of accessory cell. Sonicated extract of Actinobacillus actinomycetemcomitans suppressed interleukin-2 production of T-cells.

T helper cells have important roles in human immune system and are divided according to the production of cytokine. Th1 cells are primarily involved in macrophage-dependent immune responses, synthesize and secrete Interleukin-2 (IL-2), Interferon-γ (IFN-γ) and tumor-necrosis factor-β (TNF-β) and regulate cell-mediated immune response or delayed-type hypersensitivity. Th2 cells facilitate the synthesis of subclasses of antibody, synthesize and secrete IL-4, IL-5, IL-6, IL-13 and regulate humoral immune response. Th3 cells suppress immune response to ingested antigen and their main lymphokine is TGF-β.

In a study by Hahn et al., more lymphocytes were observed in inflamed pulps than in normal pulps, and the ratios of T4/T8 and B/T were changed with pulpal inflammation. T cells are responsible for the regulation of pulpal immunopathic changes under carious lesions.

IL-2 by Th1 cells is present in normal vital pulp and is significantly elevated in cases of symptomatic irreversible pulpitis. IL-4 by Th2 cells was detected in human periapical granulation tissue and intraosseous inflammation. In our previous study, the level of IL-2 and IL-4 was increased in experimentally induced pulpitis by S. mutans and P. endodontalis LPS. Transforming growth factor-β (TGF-β) is a key mediator of immunological homeostasis in pulp and periapical regions. TGF-β1 is of particular importance in regulating inflammation with effects that are predominantly immunosuppressive. TGF-β1 exerts potent suppressive effects on the proliferation and differentiation of both T- and B-lymphocytes.

The purpose of this study was to investigate the capacity of peripheral lymphocytes to secrete IL-2, IL-4 and TGF-β1 after stimulation with sonicated extract of E. faecalis under proper mitogenic activation.

II. Materials and methods

Preparation of bacterial sonicated extracts

Enterococcus faecalis (ATCC 29212) strains were used in this study. Strains were grown in 1-liter cultures in 85% N2-10% H2-5% CO2 chamber for 3 days at 37℃. The medium was 3.7% brain heart infusion broth supplemented with 0.5% yeast extract, 0.5 mEq L-cysteine hydrochloride, and 0.5% Sodium bicarbonate.

Bacterial fractions were prepared as previously described. Briefly, bacterial cells harvested from 1-liter cultures were washed, suspended in 20 ml of phosphate-buffered saline (PBS), to minimize the loss of protein, 1 mM of Phenylmethylsulfonylfluoride (PMSF) were added. PMSF acts as protease inhibitor at protein extraction. And Suspensions of bacterial cells were disrupted by sonication (100W output, Fisher sonicator) on ice for 5 min with 30-sec pulses-on and 10-sec pulse-off in the presence of glass beads. Disruption of the cells was confirmed microscopically. The sonicated material was centrifuged at 12000 rpm for 30min and at 30,000 rpm for 60 min. The protein that remained in suspension after high-speed centrifugation was designated the sonic extract of E. faecalis (SEF) and contained both cytoplasmic and periplasmic proteins. The supernatant was filtered with 0.22 μm syringe. The extracts was resuspended with 10-15 ml of Tris Buffer. Protein concentration was determined by the Bicinchoninic acid (BCA) protein assay (Pierce Chemical Corp., Rockford, IL, USA), and stocked in deep-freezer at -20℃.
Preparation of Human peripheral blood lymphocytes (HPBL)

HPBL were prepared from 20ml of EDTA-anticoagulated venous blood of healthy donors. The twice volume with Hanks balanced salt solution (HBSS) was added to the blood. The HPBL were isolated by buoyant density centrifugation on Ficoll-Hypaque (Phamacia LKB Biotechnology, Piscataway, N. J.; Lymphocyte separating medium). The blood was first centrifuged at 1500 rpm for 30 min at room temperature. Lymphocytes were obtained by centrifugation layered over Ficoll-Hypaque using a clean Pasteur pipette. The HPBL were washed twice with HBSS, centrifuged at 1800 rpm for 10 min at 4℃, and diluted to 2×10^6 viable cells per ml with Hematocytometer for cytokine assays. HPBL were suspended in RPMI 1640 supplemented with 100 U of penicillin/ml, 100 μg of streptomycin and 1% fetal bovine serum.

Bacterial stimulation and Cytokine production

HPBL suspension (500 μl) containing 1×10^6 cells were placed into each well of flat-bottom 24-well plate. Each culture received medium or varying concentrations of SBE diluted in medium (400 μl). The cells were then incubated for 30 min at 37℃, at which time the cultures received an optimal mitogenic dose of Phytohemagglutinin (PHA) (4 μg/ml; Sigma, 100 μl). The cells were incubated for 72 hr at 37℃ in humidified air containing 5% CO2.

Group 1: Medium only (negative control)
Group 2: 0 μg of SEF + PHA (positive control)
Group 3: 5 μg of SEF (low concentration) + PHA
Group 4: 12.5 μg of SEF (medium concentration) + PHA
Group 5: 25 μg of SEF (high concentration) + PHA

After incubation, the amount of IL-2, IL-4, TGF-β1 present in the culture supernatants were assayed by Enzyme-linked immunosorbent assay (ELISA, R & D Systems, Minneapolis, USA) according to the manufacturer’s protocols.

Statistical analysis

Data were statistically analyzed using the Mann-Whitney rank sum test and Kruskal-Wallis test. A value of p < 0.05 was considered statistically significant

### Results

The results of this study are summarized in Table 1 and Figure 1.

Bacterial extracts were evaluated for their ability to suppress the production of cytokine of lymphocyte to mitogens of PHA. Data of Table 1 and Figure 1 are the representative results of experiments. Results are the mean values of six samples of duplicate cultures. PHA-activated group

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean concentration ± SD (pg/ml protein)</th>
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<tbody>
<tr>
<td></td>
<td>IL-2</td>
</tr>
<tr>
<td>Medium only</td>
<td>17.66 ± 25.77</td>
</tr>
<tr>
<td>SEF 0 μg/ml + PHA</td>
<td>261.74 ± 75.43**</td>
</tr>
<tr>
<td>SEF 5 μg/ml + PHA</td>
<td>192.74 ± 59.81</td>
</tr>
<tr>
<td>SEF 12.5 μg/ml + PHA</td>
<td>95.05 ± 43.10*</td>
</tr>
<tr>
<td>SEF 25 μg/ml + PHA</td>
<td>51.07 ± 47.71*</td>
</tr>
</tbody>
</table>

SEF = sonicated extract of Enterococcus faecalis

"", ", " and * are statistically significant different (p < 0.05) between groups

Table 1. Average concentration (± SD) of cytokines in cultures.
(Group 2) exhibited higher level of IL-2 and IL-4 than medium only control group (Group 1) (p < 0.05). As compared with PHA-activated (Group 2), high and medium concentration of SEF (Group 4, 5) decreased the production of IL-2 and IL-4 from lymphocytes (p < 0.05), but there was no statistically significant difference between low concentration of SEF (Group 3) and PHA-activated group (Group 2). So we can suggest that SEF cause a dose-dependent reduction in PHA-induced cytokine production of IL-2 and IL-4. But the production of TGF-β1 was independent of concentration of SEF (p > 0.05).

**IV. Discussion**

Microbial products represent an important source of immunoregulatory agents. In particular, several microorganisms are capable of suppressing the immune response through various products, including toxins, enzymes, cell wall components, and metabolites. These immunosuppressive products may alter the immune system via different mechanisms. In some instances these agents indirectly modify lymphocyte response by directly affecting monocytes or macrophage activities directly. For example, Spirochetes inhibited lymphocytes function and *Leishmania donovani* impaired the synthesis and release of IL-1 from macrophages. Sonicated extract from *Prevotella intermedia*, *Porphyromonas asaccharolytica*, *Porphyromonas endodontalis* and *Prevotella melaninogena* were capable of suppressing human T- and B-cell response in dose-dependent manner. The immunosuppressive activity is nondialyzable and heat labile.

This study showed that the level of IL-2 and IL-4 in lymphocytes stimulated with PHA were significantly higher than those of the negative control group. So, PHA can have sufficient mitogenic effect to lymphocytes. But sonicated extracts of *E. faecalis* suppressed in vitro IL-2, IL-4 production of human lymphocyte in a dose-dependent manner. Ability of *E. faecalis* to impair the production of IL-2 and IL-4 may be related to the suppression of T-cell activation. In our previous studies, in contrast, lipopolysaccharide of *Porphyromonas endodontalis* in vitro stimulated the production of macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta from human polymorphonuclear leukocyte. In rat pulpitis experimentally induced by specific bacteria, *Streptococcus mutans*, *Porphyromonas endodontalis* stimulated the production of Interferon-γ, IL-2 and IL-4. These studies showed that *P. endodontalis* have inflammatory effects by stimulation, but in other reports, sonic extract of *P. endodontalis* in lymphocyte in vitro have immunosuppressive effects by inhibition, and in this study *E. faecalis* inhibited the cytokine production of lymphocytes. Much further investigation is required to clarify the stimulation and inhibition of cytokines. In contrast, there was no significant difference in the level of TGF-β1 in regardless of SEF, this results show that TGF-β1 is released by other mechanism.

When comparing with the level of each cytokine, Results showed that the level of IL-2 predominated over those of other cytokine IL-4, and TGF-β1. Considering the concentration of IL-2, Th1 lymphocytes may be dominant in human peripheral blood lymphocytes.

It has been proposed that impaired host defense may play a pivotal role in the pathogenesis of many infections. The data presented in this study...
suggest that microbial mediated immunosuppression may contribute to the pathogenesis of endodontic infection by altering the nature and consequences of host-parasite interactions. Microbial virulence may be the consequence of several properties, including the ability of certain species to resist, escape, or pervert host defense mechanisms. Some causative virus infects and destroys a subpopulation of T lymphocytes, such as human immunodeficiency virus. Such inhibitory factors could lead to a state of immunological hyporesponsiveness that favors colonization by the initiating organism or by other opportunistic organisms. Our current finding that E. faecalis inhibits the immune response suggests that impaired host defense mechanism may contribute to the disease process.

This results suggested that bacterial protein sonicated extract of E. faecalis can inhibit the immune response by lymphocyte and this could be one of possible factors why E. faecalis are found frequently in the teeth with failed endodontic treatment.

V. Conclusion

According to this study we could summarize as follows: PHA activated expression of IL-2 and IL-4 from lymphocytes compared with negative control group (p < 0.05). As compared with PHA-activated group, high and medium concentration of SEF decreased the production of IL-2 and IL-4 from lymphocytes (p < 0.05). There was no statistically significant difference between low concentration of SEF and PHA-activated group. The production of TGF-β1 was independent of concentration of SEF (p > 0.05). So we can suggest that SEF cause a dose-dependent reduction in PHA-induced cytokine production of IL-2, and IL-4.

References

국문초록

Enterococcus faecalis 추출물이 임파구의 IL-2, IL-4, TGF-β1 분비에 미치는 영향에 관한 연구

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근관치료의 실패원인 중 중요한 세균으로 알려진 Enterococcus faecalis는 최근에 중요성이 더해지며 많은 연구들이 진행중이다. 여러가지 기전들이 보고되고 있으나 면역반응에 관한 연구는 거의 알려져 있지 않은 상태이다.

본 연구에서는 Enterococcus faecalis의 초음파 분쇄 추출물을 성인의 말초혈액으로부터 얻은 임파구에 적용하여 여기서 분비되는 interleukin-2, interleukin-4, transforming growth factor-β1의 농도를 Enzyme linked immunosorbent assay (ELISA)로 측정하여 비교, 평가하는 것을 목적으로 한다.

E. faecalis를 적절한 조건에서 배양한 뒤 초음파 분쇄를 하여 추출물을 얻어냈다. 임파구는 건강한 성인의 말초혈액에서 추출하여 분리하였다. 임파구를 적절한 농도의 mitogen (Phytohemagglutinin: PHA)으로 자극시킨 뒤에 다양한 농도의 E. faecalis 초음파 추출물을 적용시키고 72시간 동안 배양하였다. ELISA를 이용하여 IL-2, IL-4, TGF-β1의 농도를 측정하였다. 실험결과는 Kruskal–Wallis test, Man–Whitney rank sum test (p < 0.05)를 사용하여 통계처리 하였다.

실험결과 PHA로 처리한 군은 아무것도 처리하지 않은 군에 비해서 IL-2, IL-4의 수치가 유의성을 있게 높았다 (p < 0.05). PHA로 처리한 군중에서 고농도와 중농도의 sonic extract of E. faecalis (SEF)로 처리한 군은 그렇지 않은 군에 비해서 IL-2, IL-4의 농도가 유의성이 있게 낮았다 (p < 0.05). PHA로 처리한 군중에서 저농도의 SEF로 처리한 군은 그렇지 않은 군과 비교하여 유의한 만한 차이를 보이지 않았다. TGF-β1의 농도는 모든 군에서 유의할 만한 차이를 보이지 않았다 (p > 0.05). 따라서, E. faecalis의 추출물은 임파구의 IL-2, IL-4의 분비능력을 저하시킨다고 할 수 있다.

주요어: Enterococcus faecalis, 임파구, 초음파 추출물, IL-2, IL-4, TGF-β1, ELISA