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ANTI-PHAGOCYTIC ROLE OF SURFACE FIBROUS STRUCTURE OF AN INVASIVE PORPHYROMONAS GINGIVALIS STRAIN

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Abstract
Recent studies have shown that invasive and non-invasive strains of Porphyromonas gingivalis can both be isolated from patients with periodontitis. We examined the interaction between an invasive 16-1 P. gingivalis strain and phagocytes obtained from human peripheral blood and guinea pig peritoneal cavity. Phagocytes from human peripheral blood, mainly polymorphonuclear leukocytes (PMNs) isolated by centrifugation in Ficoll Hypaque, and macrophages collected from the peritoneal cavity of guinea pigs, were exposed to P. gingivalis cells. After this exposure, greater numbers of the non-invasive P. gingivalis ATCC 33277 were observed in human PMNs and guinea pig macrophages compared with the invasive P. gingivalis 16-1. Electron microscopic observations showed that invasive 16-1 within phagosomes in human PMNs and guinea pig macrophages retained their surface fibrous structures as well as their outer membranes. Electron microscopic examination showed that destruction and damage to the cell membranes and inner structures were clear in human PMNs and guinea pig macrophages after exposure to invasive 16-1 for 6 and 24 hours; this was a clear difference from exposure to the non-invasive ATCC 33277. Release of lactate dehydrogenase (LDH) activities into the culture supernatant of PMNs after exposure to the invasive 16-1 for 4 and 6 hours was significantly greater than that after exposure to the non-invasive ATCC 33277 (p<0.05). On the other hand, the LDH activity after exposure for 21 hours to the invasive 16-1 was significantly lower than that of untreated cells and cells after exposure to the non-invasive ATCC 33277 strain (p<0.05). The PMN viabilities after exposure to cells of the invasive 16-1 for 3, 4, and 6 hours as evaluated by trypan blue staining were similar to those after exposure to cells of the non-invasive ATCC 33277, but that after exposure to the invasive 16-1 strain for 21 hours was significantly lower than that after exposure to cells of the non-invasive ATCC 33277 strain.

Key words: Porphyromonas gingivalis—Invasive strain—Anti-phagocytosis—Surface fibrous structure

The manuscript is the Ph.D. thesis paper of Hideaki Miyabe.
INTRODUCTION

Subgingival plaque contains various kinds of bacterial species, mainly anaerobic rods and spirochetes. Periodontitis is a disease of the supporting structures of the teeth caused by bacteria; the common ecologic niche is the gingival crevice or periodontal pocket. Porphyromonas gingivalis is considered to be an important pathogen in some forms of human periodontitis. This microorganism is particularly interesting to study because of its multiplicity of virulence factors that include those that permit adherence to host cells, gingipains that can degrade host tissues, cysteine protease, immunoglobulins, lipopolysaccharide, and those that suppress host leukocyte functions. In patients with adult periodontitis, complicated immune responses against these microorganisms have also been reported.

In the past few decades, it has been clarified that P. gingivalis strains can be classified into invasive and non-invasive strains. It has been demonstrated that the invasive strain of P. gingivalis possesses more pathogenic activity than the non-invasive strain both in vitro and in vivo. Surface components such as fimbriae and the capsule structure of P. gingivalis have been shown to be associated with adherence to host cells. However, the details of the key factors of the invasive strain are not yet elucidated. We compared the resistance of an invasive P. gingivalis 16-1 strain and a non-invasive ATCC 33277 strain against phagocytes obtained from human peripheral blood and guinea pig peritoneal cavity.

MATERIALS AND METHODS

1. Bacterial strains, growth conditions and cell preparation

An invasive 16-1 strain and a non-invasive ATCC 33277 strain of P. gingivalis were used in this study. These strains of P. gingivalis were maintained on Tryptic soy agar (Becton Dickinson and Company, Sparks, MD) supplemented with hemin (5μg/ml), menadione (0.5μg/ml), and 10% defibrinated horse blood. To adjust bacterial cell numbers, suspensions with an optical density of 0.1 at 660 nm were prepared.

2. Phagocytes obtained from human peripheral blood and guinea pig peritoneal cavity

To obtain human phagocytes, heparinized blood from a healthy adult volunteer was used. Leukocytes were isolated by centrifugation in Ficoll (Ficoll-Hypaque, Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, washed with RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) twice, and resuspended in culture medium.

Intraperitoneal phagocytes from guinea pigs, mainly macrophages, were also employed in this study. Animal experiments were conducted according to the guidelines for the treatment of experimental animals at Tokyo Dental College. To obtain intraperitoneal phagocytes from guinea pigs, 50 ml of 1% glycogen (Sigma Chemical Co., St. Louis, MO, USA) in PBS was injected into the peritoneal cavity. The intraperitoneal leukocytes were collected after 18 hours. The harvested cells were washed with RPMI 1640 medium twice and resuspended in the culture medium. The phagocyte cell numbers were adjusted to 2 × 10⁷ per ml.

3. Exposure of human PMNs and guinea pig macrophages to P. gingivalis cells

P. gingivalis cells by phagocytes were obtained from human peripheral blood and guinea pig peritoneal cavity. Each mixture of phagocytes and P. gingivalis cells was incubated by rotation at 20 times a minute in duplicate plastic tubes in an anaerobic chamber containing 10% H₂, 10% CO₂, and 80% N₂.

Fresh guinea pig serum was absorbed with both cells of P. gingivalis ATCC 33277 and 16-1 strains at 4°C for 2 hours. After centrifugation, the supernatant was filtered through a 0.2μm filter. This serum was used as a source of complement.

In each plastic tube of mixed cells, the number of phagocytes was adjusted to 5 × 10⁶,
and the number of bacterial cells was adjusted to \(1 \times 10^6\). For the opsonization assay, \(250 \mu\text{l}\) of bacterial suspension, \(250 \mu\text{l}\) of cell suspension, \(250 \mu\text{l}\) of absorbed guinea pig serum, and \(1,250 \mu\text{l}\) of RPMI 1640 medium were mixed in each tube and incubated at \(37^\circ\text{C}\) in the anaerobic chamber. After exposure for 1, 2, 4, 6 and 24 hours, an aliquot of the mixture from each tube was removed and centrifuged at \(1,500 \text{ rpm}\) for 10 minutes. The precipitate was washed with PBS twice.

4. Electron microscopic observation of phagocytes after exposure to bacterial cells

We examined the morphological changes in phagocytes in thin sections after exposure to \(P.\text{gingivalis}\) strains for 6 or 24 hours, and also observed the \(P.\text{gingivalis}\) cells in the phagocytes by transmission electron microscopy. After exposing the leukocytes to \(P.\text{gingivalis}\) cells for 6 or 24 hours, the phagocyte pellet was fixed with \(2.5\%\) glutaraldehyde in \(0.1\text{ M phosphate buffer (pH7.2)}\) for 2 hours. Samples were treated with \(1\%\) osmium tetroxide for one hour, dehydrated in a series of ethanol solutions, and embedded at low temperature. The embedding material was LowiCyrl K4M (Chemical Werke Lowi GmbH, Germany). Polymerization was done under ultraviolet light for 3 days. After ultrathin sectioning, the samples were collected on carbon reinforced formvar-coated Ni grids, stained with uranyl acetate and lead citrate, and examined in a transmission microscope (H-600, Hitachi Transmission Microscope, Tokyo, Japan).

5. Lactate dehydrogenase (LDH) activity of human polymorphonuclear leukocytes (PMNs) after exposure to \(P.\text{gingivalis}\) cells

We evaluated the cytotoxic activity of \(P.\text{gingivalis}\) strains against human peripheral leukocytes by measuring the release of lactate dehydrogenase (LDH) from the phagocytes. Seventy ml of venous blood was obtained from a healthy adult male donor. The cells were immediately separated by centrifugation at \(500 \times g\) in a swing-out rotor at \(20^\circ\text{C}\) using Polymorphrep R (Axis-Shield PoC AS, Oslo, Norway) for 30 minutes. The polymorphonuclear leukocyte (PMN) layer was carefully isolated, washed with PBS (pH7.2), and harvested following centrifugation at \(400 \times g\) for 10 minutes. The cells were then resuspended in RPMI medium 1640. The number of human leukocytes was adjusted to \(3 \times 10^5\), and the bacterial cell number was adjusted to \(6.6 \times 10^7\) in the \(500 \mu\text{l}\) reaction mixture. Two hundred \(\mu\text{l}\) of bacterial suspension, \(250 \mu\text{l}\) of phagocyte suspension, and \(50 \mu\text{l}\) of fetal calf serum were mixed in duplicate plastic tubes and rotated at 20 rpm in an anaerobic chamber. The experiment was performed by rotation at 20 times a minute in plastic tubes in the anaerobic chamber with \(10\%\) \(\text{H}_2\), \(10\%\) \(\text{CO}_2\), and \(80\%\) \(\text{N}_2\) for 3 to 21 hours. The LDH activity in the supernatant of the mixture was evaluated by using a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Pittsburgh, PA) according to the manufacturer’s instructions.

6. Viability of human peripheral leukocytes

We measured the viability of human peripheral PMNs by light microscopic observation of trypan blue staining as described previously. In the viability test of human PMNs, we used human serum absorbed with washed \(P.\text{gingivalis}\) cells of the invasive 16-1 and the non-invasive ATCC 33277 strains. The percentages of trypan blue staining cells in each sample after exposure for 6 or 24 hours were determined. Fifty leukocytes in each sample were observed, and viability was expressed as the average percentage of two tubes.

7. Statistical analysis

LDH activity was statistically analyzed by Mann-Whitney U test.

RESULTS

1. Cell surface structures of an invasive16-1 strain and non-invasive ATCC 33277 strain of \(P.\text{gingivalis}\)

Electron microscopic pictures of ultrathin...
sections of a non-invasive ATCC 33277 strain and an invasive 16-1 strain of *P. gingivalis* are shown in Fig. 1. Both strains exhibited the inner and outer membranes that are the common structures of black-pigmented anaerobic rods. Both strains also featured a distinct, electron-dense outer layer covering associated with the outer membrane. The invasive *P. gingivalis* 16-1 strain used in this study possessed a flocculent and meshwork fibrous structure that loosely adhered to its electron-dense outer layer. The non-invasive *P. gingivalis* ATCC 33277 strain possessed small amounts of thin fibrous structure, but did not have a bonded flocculent and fibrous structure.

2. **Morphological changes in phagocytes after exposure to *P. gingivalis* cells**

Thin sections of leukocytes from human peripheral blood after exposure for 6 or 24 hours are shown in Fig. 2. After exposure to the *P. gingivalis* ATCC 33277 strain for 6 or 24 hours, the thin sections of human leukocytes contained ingested bacterial cells in phagosomes.

As shown in Fig. 2, HL 16-1, 6A, some of the human peripheral leukocytes did not contain any bacterial cells after exposure to the *P. gingivalis* 16-1 strain for 6 hours. However, as indicated by arrows in Fig. 2, 16-1 24A, conspicuous erosions in the plasma membrane of leukocytes were observed after 24 hours of exposure to the 16-1 strain.

Figure 3 illustrates thin sections of macrophages from the peritoneal cavity of the guinea pig after exposure to invasive 16-1 or non-invasive ATCC 33277 cells of *P. gingivalis* for 6 or 24 hours. In these pictures, higher numbers of ingested cells of the ATCC 33277 strain than those of 16-1 strain can be observed.

As indicated by arrows in Fig. 3, GL 16-1 6B, GL 16-1 24A, and GL 16-1 24B, conspicuous erosions in the plasma membrane of the phagocytes and degenerative alterations associated with almost complete denudation were observed after 6 and 24 hours of exposure to the 16-1 strain.

3. **Morphological changes in bacterial cell surface structures in phagosomes**

Figure 4 illustrates bacterial cell surface structures of ATCC 33277 and 16-1 strains of *P. gingivalis* in phagosomes after incubation with phagocytes for 24 hours. The outer and inner membranes of both *P. gingivalis* strains in phagosomes of phagocytes obtained from either human peripheral blood or guinea pig peritoneal cavity remained as clearly identifiable structures. The distinct electron-dense outer layer of the invasive *P. gingivalis* 16-1 strain with its meshwork fibrous structure remained clear after being exposed for 24 hours, but these thick structures were not observed in the ATCC 33277 strain.
Fig. 2  Electron microscopic pictures of thin sections of human peripheral leukocytes exposed to ATCC 33277 and 16-1 \textit{P. gingivalis} strains for 6 or 24 hours. Phagocyted bacterial cells can be seen in phagosomes in many human peripheral phagocytes in thin sections. High numbers of ingested non-invasive ATCC 33277 strains can be seen in Figures HL 33277 24A and HL 33277 24B. No or low numbers of ingested cells of the invasive 16-1 strain can be seen in Figures HL 16-1 6A and HL 16-1 6B. Damage to inner and outer membrane structures in phagocytes indicated with arrows can be seen after exposure to the 16-1 strain for 24 hours in Figure HL 16-1 24A.
Fig. 3 Electron microscopic pictures of thin sections of intraperitoneal leukocytes, mainly macrophages, of guinea pig exposed to ATCC 33277 and 16-1 *P. gingivalis* strains for 6 or 24 hours. High numbers of the non-invasive ATCC 33277 *P. gingivalis* cells can be seen in phagocytes in thin sections of Figures GL 33277 6B, GL 33277 24A, and GL 33277 24B. Small numbers of the invasive 16-1 *P. gingivalis* strain can be seen in the thin section after exposure for 6 hours in Figures GL 16-1 6A and GL 16-1 6B. Conspicuous erosions of the cytoplasmic membranes of phagocytes and ruin of inner cellular structures indicated with arrows can be seen in Figures GL 16-1 6B, GL 16-1 24A, and GL 16-1 24B.
Fig. 4 Electron microscopic pictures of bacterial cells of the non-invasive ATCC 33277 and the invasive 16-1 P. gingivalis strains within phagosomes of phagocytes from human peripheral blood and peritoneal cavity of guinea pig exposed for 24 hours. Inner and outer membranes of both strains can be seen clearly. Surface flocculent, irregular electron-dense material or fibrous structure external to outer membrane of the invasive 16-1 P. gingivalis strain can be seen clearly in phagosomes of phagocytes from human peripheral blood and peritoneal cavity of guinea pig, but material external to outer membrane of the non-invasive ATCC 33277 cannot be seen.

4. LDH release from human peripheral PMNs after exposure to P. gingivalis cells

We examined the release of LDH from human peripheral PMNs after exposure to P. gingivalis cells. Figure 5 illustrates the average LDH activities of supernatants of duplicate tubes of the PMNs after exposure to P. gingivalis cells for 3 to 21 hours. Four and 6 hours after exposure to the cells of the invasive P. gingivalis 16-1 strain, the LDH release from human PMNs was significantly higher than that of exposure to the non-invasive ATCC 33277 strain (p<0.05). However, the LDH release from PMNs after exposure to the non-invasive P. gingivalis ATCC 33277 for 21 hours and non-treated cells were significantly higher than that from PMNs exposed to the invasive 16-1 strain (p<0.05).

5. Viability of phagocytes after exposure to P. gingivalis cells

Table 1 summarizes the numbers of trypan-blue-stained PMNs from human peripheral blood after exposure to bacterial cells for 3, 4, 6, and 21 hours. There were no significant differences in the viability of PMNs between the exposure to the non-invasive ATCC 33277 strain and that to the invasive 16-1 strain of P. gingivalis at 3, 4, or 6 hours. However, the viability of PMNs after exposure to the invasive 16-1 strain was significantly lower than that exposure to the non-invasive ATCC 33277 of P. gingivalis strain at 21 hours.
DISCUSSION

It has been suggested that the capsule structure of various bacterial species contributes to resistance to phagocytosis by leukocytes\(^{16,20,22}\). In this study, there were no significant differences in the viability of phagocytes as tested with trypan blue staining between those exposed to the invasive \textit{P.gingivalis} 16-1 strain and those to the non-invasive ATCC 33277 strain for 3, 4, or 6 hours. It is possible that phagocytes containing many non-invasive ATCC 33277 cells for 6 hours die from overwork in attempting to digest the bacterial cells. Sundqvist \textit{et al.}\(^{25}\) showed that invasive strains of \textit{P.gingivalis} caused spreading infections compared with the other non-invasive strains that produced small, localized abscesses. They demonstrated that there was no clear correlation between the results of the phagocytosis assay and the virulence of the bacteria when injected subcutaneously in mice. Resistance to phagocytosis may be important for the survival of these bacteria. However, this ability does not in itself imply an ability to cause damage not only to leukocytes but also to other host cells. The present study indicates that lower cell numbers of the invasive 16-1 strain were phagocytosed than the non-invasive ATCC 33277 strain by phagocytes obtained from human peripheral blood or the peritoneal cavity of guinea pig.

In the present study, we tried to assess the relative surviving activity of the invasive 16-1 strains compared with the non-invasive ATCC 33277 strain. However, we could not obtain accurate data for viable cell counting after adjusting for the optical density of both strains. Imatani \textit{et al.}\(^{8}\) in our laboratory demonstrated that the cell surface components of the invasive \textit{P.gingivalis} ATCC 53977 enhanced production of IL-6 and IL-8 from periodontal cells in vitro. The outer membrane protein and polysaccharide of invasive strains of \textit{P.gingivalis} may be pathological mediators.

In this study, the phagocytosed bacterial cells of the invasive 16-1 strain seen within the phagosomes had not lost their outer mem-

Table 1 Average percentages of trypan-blue-stained PMNs from human peripheral blood after exposure to the non-invasive ATCC 33277 and the invasive 16-1 strains of \textit{P.gingivalis}

<table>
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<th>Incubation time</th>
<th>3hr</th>
<th>4hr</th>
<th>6hr</th>
<th>21hr</th>
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<td>\textit{P.gingivalis} 33277</td>
<td>13.3</td>
<td>12.3</td>
<td>9.4</td>
<td>20.4</td>
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<tr>
<td>\textit{P.gingivalis} 16-1</td>
<td>10.1</td>
<td>13.8</td>
<td>10.4</td>
<td>60.3</td>
</tr>
<tr>
<td>None (Control)</td>
<td>6.3</td>
<td>5.8</td>
<td>7.5</td>
<td>11.6</td>
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Twenty bacterial cells per PMN were incubated in duplicate tubes, and 50 PMNs were examined in each tube.
brane or the electron-dense layer attached to the outer membrane; this suggests that their viability was not altered in the phagosomes. The electron microscopic observations showed that the invasive 16-1 strain possesses an extracellular meshwork resembling a glycocalyx, suggesting that the factor resistant to the bactericidal activity by phagocytes is the surface structure. The LDH release by the human peripheral PMNs after exposure to cells of the invasive 16-1 *P. gingivalis* at 4 and 6 hours was significantly higher than that after exposure to the non-invasive ATCC 33277 strain. However, the LDH release of the human peripheral PMNs after exposure to the non-invasive *P. gingivalis* ATCC 33277 and non-treated cells for 21 hours was significantly higher than that of PMN exposure to the invasive 16-1 strain. We found that the viability of PMNs from human peripheral blood after exposure to the invasive 16-1 strain was significantly lower than that after exposure to the non-invasive ATCC 33277 strain. It is possible that the higher amount of the LDH release arose from the death of PMNs between 6 and 21 hours after exposure to the non-invasive ATCC 33277 cells. We obtained unexpected results for the amount of LDH release and cell viability as indicated by trypan blue staining in our experiments with human PMNs after exposure to both of the *P. gingivalis* cells. In the LDH release experiment, we used human serum absorbed with both of the *P. gingivalis* cells. However, in the PMN viability test, we used calf fetal serum absorbed with both of the *P. gingivalis* cells. In addition, the increase in the LDH release from the invasive 16-1 strain exposed cells was earlier than that of the non-invasive 33277 strain exposed cells or the non-treated cells. It is possible that a part of the released LDH from the invasive 16-1 strain exposed cells was hydrolyzed by a protease of *P. gingivalis*. The delay in the peak of LDH release may induce the difference. Further studies using human serum and a more detailed time course experiment are required to elucidate the discrepancy.

Dorn et al.\(^3\) showed that the fimbriae of each *P. gingivalis* strain are implicated as critical players in invasion by this species. Using the polymerase chain reaction, *P. gingivalis* strains have been found to contain different *fimA* genes. Amano et al.\(^1\) found that more than five types of *P. gingivalis* fimbriae encoded with *fimA* are expressed on the cell surface and can be visualized by negative staining and electron microscopy. Recently, Nakagawa et al.\(^7\) demonstrated that type II FimA *P. gingivalis* strains possess strong invasive activity against host cells *in vitro*. Further, Nakagawa et al.\(^8\) reported that *P. gingivalis* with type Ib *fimA* is closely associated with the progression of periodontitis, similarly to other organisms with type II and IV *fimA*. Sequence analysis has demonstrated that the FimA types of the invasive 16-1 strain and the non-invasive ATCC 33277 are type Ib and type I, respectively, according to Amano’s typing scheme. The surface antigens of *P. gingivalis*, including the specific type of fimbriae, mediate adherence and colonization of the oral cavity by this organism and may have potential for use as vaccine antigens. One of the purposes of the present study was to determine whether *P. gingivalis* cell surface fibrous antigens have opsonic target sites. The antibody against the type Ib FimA antigen may enhance the phagocytosis and killing of *P. gingivalis* by leukocytes. We are planning to study the roles of the surface structures of *P. gingivalis*, including the fibrous structure and type Ib fimbriae, in their invasive activity into human gingival fibroblasts and vein endothelial cells.

In conclusion, the present study shows that the resistance of the invasive *P. gingivalis* 16-1 strain to phagocytes is closely related to its surface structures.

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