MICROBIAL PATHOGENESIS

Epithelial cell response to challenge of bacterial lipoteichoic acids and lipopolysaccharides in vitro

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Accumulating dental plaque at the gingival margin contains lipoteichoic acids (LTAs) from the cell walls of gram-positive bacteria. In subgingival plaque associated with periodontal disease the amount of lipopolysaccharides (LPSs) from gram-negative bacteria increases. As the gingival junctional epithelium (JE) is an important structural and functional tissue, participating in the first line defence against apical advancement of dental plaque, this study examined the direct effects of LTAs (from Streptococcus mutans and S. sanguis) and LPSs (from Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola and Escherichia coli) on two epithelial cell lines (HaCaT and ERM) and a culture model for human JE. The cells were exposed to the LTAs or LPSs (10−50 μg/ml) for variable periods of time. None of the bacterial surface components had any effect on primary adhesion or on the epithelial attachment of the JE cultures. However, cell growth and mitotic activity were consistently reduced in all cultures treated with LTAs. In contrast, LPSs showed only slight or no effects on cell growth and mitotic activity depending on the epithelial cells used. This suggests that LPSs, despite their established role as modulators of inflammation, do not have direct harmful effects - at the concentrations found in dental plaque and gingival crevicular fluid - which would explain the mechanism of epithelial degeneration and detachment from the tooth surface. However, the LTAs appear to inhibit the renewal of epithelium and may thus contribute to degeneration of coronal JE and subgingival colonisation by periodontal pathogens.

Introduction

Gingival junctional epithelium (JE) is a rapidly renewing tissue with proliferating basal cells along the connective tissue and a distinct population of proliferating cells attached to the tooth surface (DAT cells) [1]. The suprabasal location of these dividing epithelial cells is unique and appears to render them vulnerable to exogenous influences. Therefore, it seems pertinent to examine the effects that oral bacterial agents may have on epithelial cell growth and mitotic activity. Cessation of division and subsequent degeneration and detachment of the DAT cells would inevitably affect the JE barrier and expose tooth surface to apically progressing pathogenic micro-organisms. A number of metabolic end-products and structural components of bacteria have been associated with periodontal diseases [2, 3]. For example, elevated concentrations of short-chain fatty acid (SCFA) metabolites of gram-negative bacteria have been reported in plaque and gingival crevicular fluid (GCF) collected from sites exhibiting gingival inflammation [4, 5]. Interestingly, SCFAs significantly reduce epithelial cell growth and proliferation in vitro at concentrations reported in plaque and GCF [6, 7]. Also, lipoteichoic acids (LTAs) from the surface of gram-positive bacteria and lipopolysaccharides (LPSs) from gram-negative bacteria have been associated with periodontal diseases [8–12]. It has been suggested that LTAs and LPSs mediate bacterial adherence to the tooth surface and to epithelial cells [13–15]. Furthermore, bacterial surface antigens have been reported to increase epithelial permeability, penetrate healthy gingival sulcular epithelium [16, 17], cause complement activation, stimulate leucocyte function and increase cytokine production and bone resorption [10, 18–24]. In addition, different LPSs show a slight enhancement of gingival fibroblast proliferation at concentrations below 1 μg/ml, modest
to significant suppression of fibroblast proliferation at a concentration range of 10–200 μg/ml [25–29] and degeneration and death of fibroblasts at concentrations of 300–500 μg/ml [28, 30]. Both growth enhancement and growth inhibition have been described in rat epithelial cells after treatment with LPS from non-oral bacteria [31–34]. Despite the presence of both LTAs and LPSs in dental plaque in the immediate environment of the dividing JE cells [9, 12, 35–36], information on the effects of oral bacterial LPSs and LTAs on epithelial and especially on JE cells is lacking. Furthermore, as the structure of the LPSs and LTAs varies between bacterial species and also between strains [37, 38], this study investigated the effects of LTAs and LPSs of selected oral microbial species associated with different clinical conditions on epithelial culture systems commonly used in periodontal research.

Materials and methods

Cell cultures

A spontaneously transformed non-tumorigenic human skin epithelial cell line that has maintained epithelial differentiation properties (HaCaT) [39] and an epithelial cell line cultured from porcine epithelial rests of Malassez (ERM) [40] were used for this study. The cells were grown in plastic tissue-culture flasks (Nunc) in Dulbecco’s modification of Eagle’s medium (DMEM, Gibco) containing 22 mM sodium bicarbonate, 20 mM HEPES, antibiotics (penicillin G 100 IU/ml, streptomycin sulphate 100 μg/ml, amphotericin B 0.25 μg/ml) and heat-inactivated fetal calf serum (FCS) 10%. The cells were trypsinised (trypsin 0.5%-EDTA in HBSS, pH 7.2, Gibco) and divided into appropriate aliquots of cells for studies on cell adhesion, growth and proliferation.

Primary tissue culture

Masticatory mucosa was obtained, with informed consent, from four subjects undergoing a surgical exposure of impacted teeth for orthodontic reasons. The tissue was prepared and cultured to mimic JE as described previously [41]. Briefly, the tissue was cut perpendicularly to the epithelium surface into elongated fragments of c. 1 × 1 × 2 mm. The fragments were then placed on their sides on Millipore filters (pore size 0.45 μm) supported by a grid and cultured in a Petri dish in Eagle’s minimum essential medium (EMEM, Gibco) with Earle’s balanced salts solution containing 2 mM L-glutamine, 10 mM sodium bicarbonate, antibiotics (penicillin G 100 IU/ml, streptomycin sulphate 100 μg/ml, amphotericin B 0.25 μg/ml), 20 mM HEPES and heat-inactivated FCS 10%. Tissue explants were cultured for 6 days before the experiments on cell proliferation, morphology and the epithelial attachment apparatus (EAA); during this period the medium was changed every other day.

Bacterial LPSs and LTAs

LPSs from Actinobacillus actinomycetemcomitans (strain Y4), Treponema denticola (ATCC 35405) and Porphyromonas gingivalis (ATCC 33211, W 83) were purified as described previously [42]. LPS from Escherichia coli (O55:B5, L-2880) and LTAs of Streptococcus mutans (L-4152) and S. sanguis (L-3765) were purchased from Sigma. LPSs were added into the culture medium at a concentration of 50 μg/ml, LTAs at a concentration range of 10–50 μg/ml. The direct effects of LPSs and LTAs from different bacterial species on epithelial cells were compared to find out whether they caused different cellular responses.

Cell adhesion

For the studies of cell adhesion, epithelial cells (HaCaT and ERM) were seeded into microtiteration plates (96-well plates, Nunc) 2 × 10⁴ cells/well in DMEM + FCS 10% in four parallel wells. LTAs or LPSs were added at the time of starting the culture and their effects were studied after incubation for 2, 4 or 6 h at 37°C. After incubation, the medium and non-adhered cells were discarded and the adhered cells were fixed overnight in formaldehyde 4% in PBS containing sucrose 5%, washed four times with distilled water and air-dried. The cell numbers were determined after staining with crystal violet 0.1% in boric acid (200 mM, pH 6.0) at room temperature for 30 min [43, 44]. The dye bound to the cells was dissolved in equal amounts of acetic acid 10% and the absorbances were read with a Multiscan Ms Primary EIA reader with a 595-nm filter.

Cell growth

For the assessment of cell growth, epithelial cells (HaCaT, ERM) were seeded into 96-well plates (Nunc) at 1 × 10⁴ cells/well in 200 μl of DMEM containing FCS 10% and allowed to attach for 18 h in four parallel wells. Medium and unattached cells were discarded and replaced with 200 μl of medium containing LTA or LPS at a concentration range of 10–50 μg/ml. Cells were incubated at 37°C in CO₂ 5% in air for 7 days. During the incubation the medium was changed to new medium containing LTA or LPS every other day. After the culture period, the cells were fixed and stained and the number of cells in each well was determined by crystal violet staining as described above.

Cell proliferation

For studies of the direct effects of the LTAs and LPSs on epithelial cell proliferation, HaCaT and ERM cells were seeded (3 × 10⁴ cells/well) on two parallel coverslips into a 24-well plate in 1 ml of DMEM containing FCS 10%. Cells were cultured at 37°C in CO₂ 5% in air for 2 days, then the LPSs or LTAs (50 μg/ml) were added. After 2 more days in culture,
the cells were treated with 10 \( \mu \)M 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU; Sigma) for 2 (HaCaT) or 12 (ERM) h. The assay was performed according to the manufacturer's instructions for the BrdU Labelling and Detection Kit (Boehringer Mannheim Biochemica). Briefly, the cells were fixed in ethanol 70% (in 50 mM glycine buffer, pH 2) for 30 min at \(-20^\circ\)C and then incubated with mouse monoclonal antibody to BrdU containing nucleases for DNA denaturation for 30 min at 37°C. Fluorescein-conjugated anti-mouse immunoglobulin was used to identify the cells that had incorporated BrdU. Between each antibody incubation the cells were washed twice with PBS diluted with distilled water (1 in 10). The cells on the coverslips were attached on to microscope slides for examination. Four randomly selected fields of each treatment were photographed with an Aristoplan research microscope equipped with UV illumination and the ratio of labelled to non-labelled cells was counted.

A tissue-culture model for JE was used for the study of human epithelial cell proliferation in primary cultures. After 6 days in culture in EMEM, the explants were randomly placed in fresh medium without (control, \( n = 8 \)) or containing LTA (S. sanguis, \( n = 6 \); S. mutans, \( n = 6 \)) 50 \( \mu \)g/ml or LPS (T. denticola, \( n = 3 \); A. actinomycetemcomitans, \( n = 8 \); E. coli, \( n = 3 \); P. gingivalis, \( n = 10 \)) 50 \( \mu \)g/ml and cultured for a further 2 days. To examine the DNA synthesis by the epithelial cells under each culture condition, the explants were placed for 3 h in a medium to which \( ^3 \)H-thymidine (specific activity 37 MBq, 1 mCi/ml) 5 \( \mu \)l/ml was added. The explants were then rinsed with fresh medium for 30 min, fixed in glutaraldehyde 5% in 0.16 M collidine-HCl buffer (pH 7.4) for 2 days, post-fixed for 2 h in potassium ferrocyanide-reduced osmium tetroxide and embedded in epoxy resin (Glycidether 100; Merck, Darmstadt, Germany). Sections (1 \( \mu \)m thick) were cut on a Huxley LKB Ultramicrotome (LKB, Bromma, Sweden) and stained with toluidine blue for light microscopy. Step-serial sections were left unstained for autoradiography. They were coated with Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY, USA) and exposed for 8 days and developed in Kodak D-19 developer. The number of \( ^3 \)H-thymidine-labelled cells along the epithelium was counted by light microscopy.

**Morphology of epithelium and the epithelial attachment apparatus (EAA)**

For electron microscopy, ultra-thin sections from the same tissue explants were cut and stained with uranyl acetate and lead citrate and examined on a JEOL JEM-100C electron microscope (Japan Electron Optical, Tokyo, Japan). Photographs were taken and further analysed by a computer programme to compare the volume of intercellular spaces in control and in A. actinomycetemcomitans LPS-treated tissue explants, which appeared to be the only ones exhibiting a conspicuous change in this parameter.

**Statistical analysis**

The significance of the differences between treatments was tested by an analysis of variance (Statistica computer programme).

**Results**

**Cell adhesion**

None of the LTAs or LPSs studied had any direct effect on cell adhesion in either cell line studied (data not shown).

**Cell growth**

Both LTAs studied significantly inhibited HaCaT cell growth at concentration of 50 \( \mu \)g/ml after culture for 4 days (p < 0.0001, data not shown). At 7 days, the growth inhibition caused by S. sanguis LTA (50 \( \mu \)g/ml) was 28% (SD 2.5%) and by S. mutans (50 \( \mu \)g/ml) 97% (SD 0.2%), p < 0.0001. A. actinomycetemcomitans and P. gingivalis LPS increased HaCaT cell growth by 8% (SD 4%, p < 0.0001) and 12% (SD 5%, p < 0.05), respectively (Fig. 1). Neither the LPS from T. denticola nor from E. coli had a significant effect on
HaCaT cell growth. In the ERM cell line the effects of the LTAs were essentially similar to those on HaCaT cells (Fig. 2): S. sanguis LTA (50 µg/ml) reduced ERM cell growth by 42% (SD 4.8%, p < 0.0001) and the same concentration of S. mutans LTA by 82% (SD 1.4%, p < 0.0001). At 10 µg/ml, the LTAs studied did not inhibit ERM cell growth (p < 0.0001). The effects of the LPSs on ERM cells were somewhat different to those on HaCaT cells; E. coli LPS had no effect on ERM cells nor on HaCaT cell growth. A. actinomycetemcomitans, T. denticola and P. gingivalis LPSs (50 µg/ml) significantly inhibited ERM cell growth (p < 0.0001, p < 0.0001, p < 0.001, respectively). A. actinomycetemcomitans LPS caused a 35% reduction (SD 4.2%) in ERM cell growth, T. denticola LPS a 28% reduction (SD 4%) and P. gingivalis LPS a 12% reduction (SD 4.3%) (Fig. 2).

Proliferation

In HaCaT cell cultures a significant inhibition in BrdU intake was observed in cells treated with LTA from both S. sanguis and S. mutans (p < 0.0001) (Table 1). During the BrdU labelling period of 2 h, 54% of the control cells incorporated BrdU label (ratio 1.16), as did 45% of the LTA-treated cells (ratios 0.86, 0.82). When HaCaT cells were treated with the different LPSs, no significant effects on cell proliferation were observed (Table 1). In ERM cell cultures, both streptococcal LTAs and LPSs from the pathogens studied significantly inhibited BrdU-intake (p < 0.0001, p < 0.005, respectively) (Table 2). In the untreated primary human explant cultures, the average number of DNA-synthesising epithelial cells inbetween the connective tissue and the filter was 4.26 (SD 2.76)/mm of the epithelium (Fig. 3a,b). Treatment of the explants with LTA from S. sanguis caused a significant reduction in epithelial cell proliferation (p < 0.05, Table 3). A reduction in the number of proliferating cells was also observed in cultures treated with S. mutans LTA and P. gingivalis LPS; however, it did not reach statistical significance. Cells treated with A. actinomycetemcomitans, T. denticola or E. coli LPSs did not significantly differ from controls (Table 3).

Electron microscopy

The examination of the JE culture model by electron microscopy showed that in A. actinomycetemcomitans LPS-treated explants, widened intercellular spaces were seen through the whole thickness of the explant epithelium. The intercellular spaces occupied 4.36% of the epithelial volume in control explants and 14.55% in A. actinomycetemcomitans LPS-treated explants (Fig. 4a,b,c,d). Explants treated with other LPSs or LTAs had similar morphology to the controls. All explants exhibited normal-looking epithelial cells attached to the substratum with an intact EAA (Fig. 4b,d,e,f). No signs of degenerative vacuolisation observed in DAT cells collected from periodontally involved teeth in vivo [45] were seen (Fig. 4).

Table 1. BrdU-labelling of HaCaT cells treated with LTA or LPS (50 µg/ml) for 2 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average ratio of labelled: unlabelled cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control for LTAs</td>
<td>1.16</td>
<td>0.06</td>
</tr>
<tr>
<td>S. sanguis LTA</td>
<td>0.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08</td>
</tr>
<tr>
<td>S. mutans LTA</td>
<td>0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12</td>
</tr>
<tr>
<td>Control for LPSs</td>
<td>1.17</td>
<td>0.17</td>
</tr>
<tr>
<td>A. actinomycetemcomitans LPS</td>
<td>1.14</td>
<td>0.23</td>
</tr>
<tr>
<td>P. gingivalis LPS</td>
<td>1.02</td>
<td>0.18</td>
</tr>
<tr>
<td>T. denticola LPS</td>
<td>1.07</td>
<td>0.09</td>
</tr>
<tr>
<td>E. coli LPS</td>
<td>1.17</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Four parallel fields.
<sup>p</sup> < 0.0001.
<sup>a</sup> < 0.0001.
<sup>b</sup> < 0.0001.
<sup>c</sup> < 0.0001.
<sup>d</sup> < 0.0001.
<sup>e</sup> < 0.0001.
<sup>f</sup> < 0.0001.

Table 2. BrdU-labelling of ERM cells treated with LTA or LPS (50 µg/ml) for 2 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average ratio of labelled: unlabelled cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.74</td>
<td>0.36</td>
</tr>
<tr>
<td>S. sanguis LTA</td>
<td>0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.14</td>
</tr>
<tr>
<td>S. mutans LTA</td>
<td>0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
<tr>
<td>A. actinomycetemcomitans LPS</td>
<td>0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09</td>
</tr>
<tr>
<td>P. gingivalis LPS</td>
<td>0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.19</td>
</tr>
<tr>
<td>T. denticola LPS</td>
<td>0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08</td>
</tr>
<tr>
<td>E. coli LPS</td>
<td>0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> Four parallel fields.
Gingival JE is a tissue with dividing cells, not only in the basal layer, but also suprabasally along the tooth surface [1, 46–48]. Inflammation stimulates the basal cells of JE leading to epithelial sprouting into the connective tissue [49]. However, recently an increasing amount of evidence has suggested that although epithelial cell division is stimulated by inflammation to a certain degree, inhibition and subsequent degeneration and cell death take place when the adaptive threshold is exceeded [45, 50, 51]. In JE, the location of dividing DAT cells away from the connective tissue and blood circulation may (as a result of compromised nutritional conditions, for example) impair their ability to adapt to changes caused by the inflammatory reaction or to external challenges.

Bacterial surface antigens, LTAs and LPSs are released by plaque bacteria at a concentration range of 0.4 ng-50 µg/ml [8, 9, 12, 35]. The aim of the present work was to examine whether these bacterial agents interfere with the adhesion, attachment, growth and mitotic activity of epithelial cells, thus suggesting a role for these molecules in the degeneration of junctional epithelial cells that is associated with periodontal diseases. For this study, concentrations of 10–50 µg/ml of the LTAs and LPSs were chosen to resemble the concentrations to which the epithelial cells may be exposed in vivo in the sulcular region and that are not yet toxic to cells. A human gingival explant culture model was used to achieve an experimental set up as close as possible to the situation in vivo at the dentogingival junction. Two established epithelial cell lines [39, 40] were used because they are well characterised, easy to grow and widely used in other laboratories. Furthermore, an advantage of established cell lines is that the possibility of host-specific variation in response to a given bacterial challenge is eliminated, a possibility to be considered especially regarding the LPSs [52, 53].

To study the possible role of LTAs in the initial phase of sulcus formation, streptococcal LTAs from two oral species (S. sanguis and S. mutans) were included. The study showed that in all the different cell cultures both of the streptococcal LTAs used at concentrations of 50 µg/ml were potent inhibitors of epithelial cell growth and proliferation. This is a novel finding and suggests that the initial colonisers on the tooth surface are potentially able to affect JE cell proliferation. As even the most coronal DAT cells attached to the tooth surface have been shown to be dividing, the LTAs may have a role in degeneration and subsequent detachment of this population of cells from the tooth surface. Streptococcal LTA production is known to be enhanced in alkaline pH [54] and elevated pH values prevail in plaque and in the GCF of gingivitis and periodontitis patients [55, 56]. Therefore, local environmental factors may favour LTA production and thus contribute to epithelial degeneration and exposure of the tooth surface. The formation of a deepened gingival sulcus may either lead to an equilibrium or further contribute to subgingival colonisation by anaerobic pathogens. Clearly, the importance of the immunological defence of the host is emphasised in sites of deepening sulci.

LPSs from parodontopathogens A. actinomycetemcomitans, T. denticola and P. gingivalis were included in the study to examine the possible role of these cell-surface antigens on epithelial pathology and on the progression of periodontal pocket formation. E. coli LPS was included to serve as a reference to the LPSs from periodontal pathogens used in this study and to

Discussion

Gingival JE is a tissue with dividing cells, not only in the basal layer, but also suprabasally along the tooth surface [1, 46–48]. Inflammation stimulates the basal cells of JE leading to epithelial sprouting into the connective tissue [49]. However, recently an increasing amount of evidence has suggested that although epithelial cell division is stimulated by inflammation to a certain degree, inhibition and subsequent degeneration and cell death take place when the adaptive threshold is exceeded [45, 50, 51]. In JE, the location of dividing DAT cells away from the connective tissue and blood circulation may (as a result of compromised...
previous studies that used E. coli LPS. It was found that 50 \( \mu g/ml \) of the LPSs studied had different effects on cell growth and proliferation depending on the type of epithelial cells used. HaCaT cell growth was slightly increased after treatment with A. actinomycetemcomitans and P. gingivalis LPSs for 7 days compared with controls. This increase did not appear to be due to changes in the mitotic activity of the cells. However, it is possible that the mitotic activity of the cells is enhanced after a longer period of exposure than that used in the BrdU assay (2 days) and is, therefore, seen only in the 7-day growth assay. Alternatively, the explanation for the slight increase in cell growth may be due to an increase in cell volume, as the crystal violet staining measures the dye absorbed into cells. Unlike the HaCaT cell cultures, ERM cell growth and proliferation were significantly inhibited by the LPSs of periodontal pathogens. In primary explant cultures the LPSs studied had no effect on the mitotic activity of epithelial cells. In general terms, the observed varying effects of LPSs on cell growth and proliferation are consistent with previous studies, showing growth enhancement or growth inhibition depending on the cell line studied. An inhibition of epithelial cell growth was reported when approximately the same LPS concentration (6–50 \( \mu g/ml \)) as that used in the present study was used [32, 33].

Epithelial stimulation by LPSs has been reported to be significant only at concentrations from 200 \( \mu g/ml \) to 5000 \( \mu g/ml \) [31, 34].

In conclusion, when compared to the effects of the LTAs shown in this study and to the previously reported effects of the SCFA metabolites of gram-negative anaerobes [6, 7] on human epithelial cells, the direct effects of the LPSs shown here seem to be relatively weak – with the exception of A. actinomycetemcomitans LPS, which increased the intercellular spaces of the epithelium. Therefore, it seems that the

Fig. 4. Electron micrographs showing the epithelial cell–filter interface (arrows). (a) and (b) Control explants, (c) and (d) A. actinomycetemcomitans LPS-treated explants, (e) P. gingivalis LPS-treated explant, (f) S. mutans LTA-treated explant. No obvious differences are seen in the number or structure of hemidesmosomes (HD) of the EAA expressed by the DAT cells of the in-vitro model of junctional epithelium. In A. actinomycetemcomitans LPS-treated explants (c,d), widened intercellular spaces (+) are seen on the whole depth of the explant epithelium. N, nucleus of the cells; F, growth filter. Bar = 5 \( \mu m \) in (a) and (c); bar = 1 \( \mu m \) in (b), (d), (e) and (f).
effects of the LPSs are directed mainly towards the inflammatory reaction in the connective tissue rather than the impairment of epithelial cell function. However, it has been shown recently that inflammatory cytokines are hundreds of times more potent in stimulating inflammatory mediator (IL-6) production by fibroblasts than LPSs [57]. The concordance of these sets of results supports the need to critically examine the potency and relative importance of LPSs as pathogenic components in the clinical environment of the JE cells. In contrast, the LTAs of the initial colonisers appear to harmful to the vital functions of epithelial cells and thus may influence the balance between the external challenge and host defence at the dentogingival junction.

References


