Interaction of extracellular vesicles of *Bacteroides gingivalis* W50 with human polymorphonuclear leucocytes

Helen M. Kay, A.J. Birss and J.W. Smalley

Department of Clinical Dental Sciences, The University of Liverpool, Liverpool, U.K.

Received 4 June 1990
Accepted 11 June 1990

Key words: *Bacteroides gingivalis*; Neutrophil; Virulence

1. SUMMARY

The effects of *B. gingivalis* W50 extracellular vesicles (ECV) on neutrophil chemotaxis and viability were assessed and compared with those of whole cells and the extracellular non-dialysable soluble protein (EP) fraction. None of the fractions tested, including soluble fractions derived from cells and ECV by sonication, induced neutrophil chemotaxis. Only ECV and cells inhibited f-MLP-stimulated chemotaxis. ECV and cells were cytotoxic towards neutrophils. The cytotoxic response was time dependent. The soluble EP fraction did not influence cell viability.

2. INTRODUCTION

Polymorphonuclear leucocytes (PMNLs) are vital in the host’s first line of defence against bacterial infection. The major type of PMNLs are neutrophils and these cells are characterized by their ability to migrate not only randomly, but also in response to a chemical gradient; a phenomenon known as chemotaxis. In periodontal disease neutrophils accumulate in the periodontal pocket, junctional epithelium and around the blood vessels of the gingival connective tissue [1]. There is considerable evidence that neutrophils play an important protective role in periodontal disease [2]. Depressed or defective neutrophil function or numbers are associated with an increase in the incidence and severity of periodontal diseases [3]. The ability of an organism to prevent or depress the neutrophil response could therefore constitute an important virulence factor.

*Bacteroides gingivalis*, which has been strongly implicated in the pathogenesis of periodontal disease, possesses a variety of putative virulence factors including proteolytic enzymes, lipopolysaccharide and extracellular vesicles [4]. The aim of this study was to investigate the effect of extracellular vesicles on neutrophil chemotaxis, chemotaxis inhibition and neutrophil viability.

3. MATERIALS AND METHODS

3.1. Bacterial growth

*Bacteroides gingivalis* strain W50 was employed and grown as previously described [5] in 1 l batch...
cultures and harvested at the end of log phase growth (48 h). Cells were removed by centrifugation (10,000 × g for 30 min) and contaminating growth medium constituents were removed by washing twice in 0.01 M sodium phosphate buffered saline (0.14 M NaCl, pH 7.4 (PBS), and once in deionized water, and freeze-dried to constant weight.

3.2. Preparation of bacterial fractions

All manipulations were carried out at 4°C. Extracellular vesicles (ECV) were prepared from the cell-free growth medium supernatant [6]. This also yielded a vesicle-free extracellular protein (EP) fraction. For some experiments, ECV and whole washed bacterial cells were disrupted by sonication in PBS [7] at concentrations of 2 and 4 mg ml⁻¹ respectively. The soluble supernatant fractions produced by this step from either cells or ECV (designated cell-SF and ECV-SF) were separated from the insoluble membrane fractions by centrifugation at 75,000 × g for 1 h. The protein content of these fractions was determined by the Folin-phenol method using bovine serum albumin as standard.

3.3. Isolation of neutrophils

Human neutrophils were isolated from fresh heparinized human blood by the combined dextran/Ficoll sedimentation method of Hasten and Shields [8]. Cell pellets obtained after centrifugation through Ficoll were washed in Hank's balanced salts solution (HBSS) containing 10 mM MOPS, pH 7.3, and contaminating erythrocytes removed by hypotonic lysis in distilled water. Neutrophils were resuspended in HBSS/MOPS to the required concentration and used immediately.

3.4. Neutrophil chemotaxis assay

Induction and inhibition of chemotaxis was investigated using the technique of migration under agarose [9]. Sets of 3 mm diameter wells, 3 mm apart were cut in 2.5 mm deep agarose dissolved in Eagle's MEM containing 10 mg ml⁻¹ human serum albumin. The chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (f-MLP) (10⁻⁷ M) in HBSS/MOPS was used as positive control.

3.5. Induction of chemotaxis

The chemotactic response of neutrophils (10⁵ per well) was tested against 11.5 µl aliquots of cells, ECV and EP (at 1000, 10, 0.1 and 0.01 µg ml⁻¹) and ECV-SF (300, 3, 0.03, and 10⁻⁴ µg protein ml⁻¹). Plates were incubated for 2 h at 37°C in a humidified atmosphere containing 4.5% CO₂, and then fixed in methanol and 10% buffered formal saline. The migration distances of adherent neutrophils were measured to the nearest 0.1 mm with a microscope graticule after staining with Wright's stain.

3.6. Inhibition of f-MLP-stimulated chemotaxis

Neutrophils (10⁶ ml⁻¹) were incubated for 1 h at 37°C with equal volumes of cell, EP and ECV suspensions (each 1 mg ml⁻¹; 425, 210 and 423 µg protein ml⁻¹), ECV-SF (300 µg protein ml⁻¹) and cell-SF (1000 µg protein ml⁻¹). The chemotactic response to f-MLP was then tested as above.

3.7. Cytotoxic assays

(a) Trypan blue exclusion. Neutrophils (10⁶ ml⁻¹) in 199 medium were exposed to equal volumes of cells, EP and ECV samples (1 mg dried weight ml⁻¹) for 1 or 3 h at 37°C. Cell viability was assessed by proportional counts after staining with 0.1% (w/v) trypan blue.

(b) MTT assay. This colorimetric method [10] involves the intracellular conversion of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into the blue coloured formazan by viable cells. Neutrophils were exposed to bacterial cell or extracellular samples as above for 1 h at 37°C. The neutrophils were then pelleted by centrifugation, resuspended in 199 medium and 200 µl aliquots incubated with MTT (50 µg ml⁻¹) for 3 h at 37°C. After centrifugation the supernatant was discarded and the formazan in the cell pellet dissolved in 50 µl ethanol. The absorbance was measured in microtest plates at 570 nm with a 630 nm reference absorbance. Percentage viability was calculated by reference to absorbances produced by neutrophils (10⁵) incubated with MTT alone.
4. RESULTS

4.1 Induction of neutrophil chemotaxis

The particulate fractions, i.e. cells and ECV, over a range of concentrations between 1000 and $10^{-3}$ µg ml$^{-1}$, consistently failed to induce chemotaxis (Table 1). Over the same concentration range, neither the soluble EP fraction nor ECV-SF could induce a chemotactic response.

4.2 Inhibition of f-MLP-stimulated chemotaxis

As shown in Table 2, incubation with cells and ECV (at a final concentration of 500 µg ml$^{-1}$) inhibited f-MLP-stimulated chemotaxis as compared to the control, in which neutrophils were preincubated with PBS only. In contrast, however, preincubation of neutrophils with the soluble fractions EP, cell-SF and ECV-SF gave no depression of the chemotactic response, the chemotactic differentials for these neutrophils being similar to those incubated with PBS. However, the reduction in both the response towards f-MLP and in spontaneous migration observed in the inhibition as compared to the induction experiments may be attributable to the extended time period of this assay.

![Table 1](attachment:image.jpg)

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chemotactic differential (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>Cells</td>
<td>0.0</td>
</tr>
<tr>
<td>Controls: PBS</td>
<td>0.0</td>
</tr>
<tr>
<td>f-MLP</td>
<td>2.2</td>
</tr>
<tr>
<td>ECV</td>
<td>0.0</td>
</tr>
<tr>
<td>Controls: PBS</td>
<td>0.0</td>
</tr>
<tr>
<td>f-MLP</td>
<td>1.0</td>
</tr>
<tr>
<td>EP</td>
<td>0.0</td>
</tr>
<tr>
<td>Controls: PBS</td>
<td>0.0</td>
</tr>
<tr>
<td>f-MLP</td>
<td>1.1</td>
</tr>
<tr>
<td>ECV-SF</td>
<td>0.1</td>
</tr>
<tr>
<td>Controls: PBS</td>
<td>0.0</td>
</tr>
<tr>
<td>f-MLP</td>
<td>1.0</td>
</tr>
</tbody>
</table>

4.3 Cytotoxicity assays

Fig. 1 shows the effect of B. gingivalis W50 cellular and extracellular fractions on neutrophil viability as assessed by trypan blue exclusion. Exposure of neutrophils for 1 h to EP and cell-SF resulted in a low mortality (3%) similar to negative

![Diagram](attachment:image.png)

Fig. 1. Cytotoxic response of isolated neutrophils to (1) ECV, (2) cells, (3) EP, (4) cell-SF and (5) control fractions as assessed by trypan blue exclusion after (a) 1 h and (b) 2 h exposure. Data points represent the average percent mortality (and range) of three replicate determinations.
Table 2

Chemotactic differentials of f-MLP stimulated neutrophils after exposure to B. gingivalis W50 fractions

All sample concentrations were 1 mg ml⁻¹ except cell-SF and ECV-SF (1000 and 300 µg protein ml⁻¹). The averages are from 3 determinations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chemotactic differential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>ECV</td>
<td>0.0</td>
</tr>
<tr>
<td>EP</td>
<td>0.9</td>
</tr>
<tr>
<td>PBS</td>
<td>0.8</td>
</tr>
<tr>
<td>Cells</td>
<td>0.0</td>
</tr>
<tr>
<td>Cell-SF</td>
<td>0.8</td>
</tr>
<tr>
<td>ECV-SF</td>
<td>0.8</td>
</tr>
<tr>
<td>PBS</td>
<td>0.9</td>
</tr>
</tbody>
</table>

controls (Fig. 1a). The percentage mortalities for whole cells and intact ECV were 12 and 13 respectively. When exposure was extended to 3 h (Fig. 1b), the intact cells caused almost complete (94%) mortality, although ECV-induced mortality was only increased to 22%. The increased exposure time had little effect in the case of EP and cell-SF.

The cytotoxic effect of ECV and cells was confirmed with the MTT assay (Fig. 2). The EP fraction had little influence on viability whilst ECV and cells effected approximately 50% mortality. The cell-SF fraction caused 30% mortality. These differences may be accounted for by the extended exposure time of neutrophils to bacterial fractions in this assay.

5. DISCUSSION

This study has confirmed previous reports of the inability of whole cells of B. gingivalis to stimulate neutrophil chemotaxis. However we have specifically shown that neither the particulate ECV nor the non-dialysable soluble extracellular protein fractions recoverable from culture supernatants of strain W50 are capable of inducing a chemotactic response. These results are not wholly unexpected since other groups [11,12] using the Boyden chamber and migration under agarose methods have shown B. gingivalis culture supernatants to be non-chemotactic. It is noteworthy that neither the cell- nor ECV-SF could induce chemotaxis. The presence of B. gingivalis cells and extracellular vesicles (or their soluble products) in the gingival crevice may not therefore stimulate a neutrophil influx. The recruitment of such cells into gingival exudate and tissues is possibly mediated by other plaque components with known in vitro chemotactic activity [13]. However, chemotaxis may be induced indirectly by plaque bacteria, including B. gingivalis, through complement activation [14,15].

The inhibition of f-MLP stimulated chemotaxis by B. gingivalis culture supernatants has been previously reported [11]. In this report inhibition was attributed to dialysable low molecular mass components, thought to compete for f-MLP receptors on the neutrophil surface. This might explain the lack of inhibition by the EP fraction since it was extensively dialysed in its preparation. However, the extracellular inhibitory macromolecular components of strain W50 reside in the ECV fraction. Van Dyke et al. [11] also showed inhibition of endotoxin-activated serum-stimulated chemotaxis by B. gingivalis culture supernatant, and since these components in this system bind to different surface receptors, it raises the possibility that inhibition results from a general interference with chemotactic receptors. This is supported by the observations of Maeda et al. [16], who found that culture supernatants could
suppress not only f-MLP binding but also f-MLP-stimulated superoxide anion production. The active components were not identified but it is possible that these fractions contained ECV. We have preliminary evidence that ECV do not elicit neutrophil reactive oxidant generation. This would indicate that vesicles are not readily phagocytosed and thus may exert inhibitory effects at the neutrophil surface.

The ECV and whole cells displayed cytotoxic activity towards isolated neutrophils. Cellular and extracellular fractions of *B. gingivalis* are claimed to be non-leucotoxic [11,17]. However, exposure times of neutrophils to these fractions may be critical since both above groups employed much shorter exposure times than our study. The cytotoxic mechanism has yet to be elucidated. The ability of cells or ECV to immobilise neutrophils could greatly facilitate the cytotoxic process.

Chemotaxis inhibition and cytotoxic activity of ECV and cells toward neutrophils may enable them to resist this facet of the host response. This may facilitate their persistence in the gingival crevice and surrounding tissues. These findings lend further support for the potential role of extracellular vesicles in the virulence of *B. gingivalis*.

REFERENCES


