Adherence of *Fusobacterium necrophorum* to bovine ruminal cells

M. KANOE and K. IWAKI

*Department of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, 753 Yamaguchi-shi, Japan*

**Summary.** The adherence of *Fusobacterium necrophorum* to the surface of bovine ruminal epithelial cells was paralleled by the organism’s haemagglutinating ability. Treatment of the bacterial cells with haemagglutinin antiserum caused a reduction in the degree of attachment. The purified haemagglutinin became bound to the membranes of ruminal epithelial cells but lost its adherence when pre-incubated with haemagglutinin antiserum. These findings suggest that the adherence of *F. necrophorum* to the membrane of the ruminal cells is mediated by haemagglutinin.

**Introduction**

Bacterial adherence to mucosal surfaces is recognised as an important early step in infection (McNeish *et al.*, 1975; Beachey, 1981). The ability of certain pathogens to attach themselves to mammalian cells appears to be mediated by bacterial pili, haemagglutinin (HG) and capsule (Burrows *et al.*, 1976; Wilson and Collier, 1976; Fader *et al.*, 1979; Tajima *et al.*, 1985). It is thought that *Fusobacterium necrophorum* colonises the bovine ruminal epithelium, sometimes penetrating into the venous system and thereby infecting the abdominal organs (Kanoe *et al.*, 1984).

Little is known, however, about the mechanism of adherence and mucosal colonisation. Previously we described the purification of *F. necrophorum* HG (Nagai *et al.*, 1984). In this paper we report the adherence of *F. necrophorum* to bovine ruminal epithelial cells and the role played by HG.

**Materials and methods**

**Bacterial strains and culture**

Four strains were used in this study. Two (VPI 2891 and 118) were kindly supplied by Dr Shinjo, Miyazaki University and one (S-45) by Dr Miwatani, Osaka University. Strain "Goat" was isolated from a caprine oral abscess. As described by Nagai *et al.* (1984), bacteria were cultured at 37°C for 24 h in modified heart infusion broth supplemented with cysteine monohydrochloride 0·1% and ascorbic acid 0·1%. They were then collected by centrifugation, washed three times with phosphate buffered saline (PBS, pH 7·2), and resuspended in PBS to a concentration of 10⁷ cells/ml.

**Preparation of cells**

Bovine ruminal and reticular tissue was obtained from an abattoir. Epithelial cells, collected by gentle brushing with a toothbrush, were washed three times with PBS and resuspended in the buffer to a density of 10⁵ cells/ml.

**Adherence assay**

The epithelial cell suspension (1·0 ml) and the bacterial cell suspension (0·3 ml) were mixed and incubated at 37°C for 1 h in a water bath. The mixture was then lightly centrifuged and resuspended three times in PBS to remove unattached bacteria. The final sediment was resuspended in 1·0 ml of PBS and one drop of the suspension was smeared over a glass slide. The smear was fixed with methanol 95% v/v for 5 min, washed, and stained with Giemsa's stain. It was then examined by light microscopy (x 400). Usually 30 cells/slide were studied; the proportion of cells with bacteria attached to the surface and the number of bacteria/cell were counted.

**Haemagglutination test**

The method was as described previously (Nagai *et al.*, 1984). Briefly, the bacterial suspension (50 μl) was diluted serially in doubling dilutions in the wells of a plastic tray. An equal volume of suspension (1·0%) of chicken RBC was added to each well. After incubation at 37°C for 30 min and room temperature for 1 h the test was read, the titre being indicated by the highest dilution showing complete haemagglutination.
Preparation of HG and HG antiserum

HG (Nagai et al., 1984) was prepared as follows. To 5 ml of the bacterial cell suspension (10^7 cells/ml), 0.1 g of trypsin (1 in 250; Difco Laboratories, Detroit) was added and the mixture was held at 37°C for 1 h. The trypsin was removed by washing twice with a mixture of 0.15 M NaCl and 10 mM Tris (hydroxymethyl) aminomethane hydrochloride buffer at pH 7.0 (TBS). The cells were resuspended in TBS containing cysteine HCl 2% and ascorbic acid 2% before being incubated in a water bath at 37°C for 1 h and then disrupted with a Super Sonic Vibrator (VR 150 P, Tomy Seiko Co., Tokyo) for 5 min with 30-s bursts separated by intervals of 2 min. The suspension was centrifuged at 10,000 g for 30 min at 4°C and the supernate filtered through a membrane filter (Millipore Corp., Bedford, MA, USA) with a pore diameter of 0.6 μm. The filtrate was then purified by Sephadex G-100 (Pharmacia, Uppsala, Sweden) column chromatography. The purified HG had a protein content of 40 μg/ml. HG antiserum was prepared in rabbits by four intramuscular injections (1.0 ml), at weekly intervals, of purified HG emulsified with an equal volume of Freund's complete adjuvant. Serum was collected one week after the final injection and stored at −20°C.

Adherence inhibition test

Bacterial suspension (50 μl; 10^7 organisms/ml) was added to an equal volume of the homologous HG antiserum (diluted 1 in 10 in PBS). After incubation at 37°C for 30 min, the mixture was added to the epithelial-cell suspension (10^6 cells/ml), allowed to stand at 37°C for 30 min, and washed with PBS. The sediment was resuspended in 0.2 ml of PBS and one drop was smeared over a glass slide. As described above, the smear was dried, fixed, stained, and examined by light microscopy. Inhibition was expressed as the ratio of the number of antiserum-treated bacteria/cell to the number of untreated bacteria/cell.

Pretreatment of bovine epithelial cells

Trypsin, pepsin (Worthington Diagnostic Systems Inc., Freehold, NJ, USA) and lipase (Wako Chemicals, Tokyo) were used. Cell suspension (1 ml; 10^6 cells/ml) was mixed with 2 ml of the pretreatment solution (0.5 μg/ml) and allowed to stand at 37°C for 30 min. The cells were washed and resuspended in PBS. Bacterial adherence was then assessed as described above.

Immunofluorescence

The epithelial cell suspension (1 ml) was mixed with 2 ml of the purified HG, incubated at 37°C for 1 h, washed with PBS, resuspended in a small volume of PBS, and smeared on a glass slide. The smear was dried, fixed with acetone for 10 min, and exposed to a 1 in 160 dilution of homologous HG antiserum at 37°C for 20 min. After washing it was exposed to a 1 in 40 dilution of fluorescein isothiocyanate-conjugated goat antiserum to rabbit IgG at 37°C for 1 h. After washing with PBS, the cells were examined by fluorescence microscopy. In addition, the purified HG (2 ml) was pre-incubated with the homologous antiserum (2 ml) at 37°C for 1 h. The mixture (2 ml) was then employed in the immunofluorescence study as mentioned above.

Scanning electronmicroscopy

The epithelial and bacterial cell suspensions were mixed and incubated at 37°C for 1 h. The mixture was then fixed in glutaraldehyde 2% in 0.1 M cacodylate buffer, pH 7.2, dehydrated in a critical point apparatus (JFC-110; Japan Electron Optics, Tokyo) and, after a gold evaporation step, examined with a scanning electronmicroscope (JSM-25SIII; Japan Electron Optics, Tokyo) at an operating voltage of 25 kV.

Statistical analysis

Statistical significance among means was determined by the χ²-square test. A p-value of 0.05 or less was reported as significant.

Results

Adherence and haemagglutinability

All four strains of F. necrophorum showed adherence to the surface of bovine ruminal cells and three of them possessed haemagglutinating activity. As shown in table I, strains VPI 2891 and Goat had a strong affinity for the cell surface (p < 0.01) and showed a high haemagglutinating activity, whereas strains S-45 and 118 adhered less well, and demonstrated little or no haemagglutinating activity. Attachment of the strains to reticular epithelial cells was similar to that of ruminal cells. Strain VPI 2891 was employed in the following tests.

Table I. The haemagglutinability of F. necrophorum and adherence to bovine ruminal cells

<table>
<thead>
<tr>
<th>F. necrophorum strain</th>
<th>Mean number of adherent bacteria/cell (SD)</th>
<th>Haemagglutination titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPI 2891</td>
<td>16-1 (1-2)*</td>
<td>128</td>
</tr>
<tr>
<td>S-45</td>
<td>5-0 (0-8)</td>
<td>0</td>
</tr>
<tr>
<td>118</td>
<td>7-4 (1-2)</td>
<td>8</td>
</tr>
<tr>
<td>Goat</td>
<td>16-5 (1-7)*</td>
<td>64*</td>
</tr>
</tbody>
</table>

SD = standard deviation of triplicate determinations.
* = Significantly different (p < 0.01) from the lower values.
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**Scanning electronmicroscopy**

The fusobacteria attached themselves to the surface of the ruminal epithelial cells at 37°C (fig. 1) but did not become surrounded by microvilli of the cell membrane.

**Adherence inhibition**

Adherence was strongly inhibited by homologous HG antiserum in a dose-dependent manner. Normal rabbit serum had a similar but weaker inhibitory effect (table II).

**Immunofluorescence**

Fluorescence specific for the HG was demonstrated on the surface of HG-treated bovine ruminal cells.

![Fig. 1. Scanning electronmicrograph of bovine ruminal epithelial cells with adherent *F. necrophorum* strain VPI 2891. Bar indicates 5 μm.](image)

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antiserum diluted 1 in</th>
<th>Mean number of adherent bacteria/cell (SD)</th>
<th>Inhibition of adherence by HG antiserum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (none)</td>
<td>...</td>
<td>40.9 (2.7)</td>
<td>100</td>
</tr>
<tr>
<td><em>F. necrophorum</em> haemagglutinin antiserum</td>
<td>5</td>
<td>3.9 (0.3)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13.9 (0.3)</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>20.6 (0.8)</td>
<td>51</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>5</td>
<td>21.2 (0.6)</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>27.6 (0.6)</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>36.6 (0.5)</td>
<td>90</td>
</tr>
</tbody>
</table>

SD = as in table I.
* Number of antiserum-treated bacteria/cell × 100.

**Table III. Inhibition of adherence by pretreatment of bovine ruminal cells**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Number of adherent bacteria/cell (SD)</th>
<th>Inhibition of adherence by pretreatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>56.7 (0.6)</td>
<td>100</td>
</tr>
<tr>
<td>Pepsin 0.5 mg/ml</td>
<td>28.5 (2.7)†</td>
<td>50.3</td>
</tr>
<tr>
<td>Trypsin 0.5 mg/ml</td>
<td>37.8 (3.5)†</td>
<td>66.7</td>
</tr>
<tr>
<td>Lipase 0.5 mg/ml</td>
<td>49.1 (2.8)</td>
<td>86.6</td>
</tr>
</tbody>
</table>

SD = as in table I.
* Number of pretreated bacteria/cell × 100.
† Significantly different (p < 0.01) from the control.
Fig. 2. Indirect fluorescent staining of bovine ruminal epithelial cells treated with (a) the HG of F. necrophorum, and (b) HG pre-incubated with HG antiserum. Bar indicates 50 μm.

Pretreatment of bovine ruminal cells

Pretreatment of the bovine ruminal cells with pepsin or trypsin caused a reduction in the number of adherent bacterial cells of about 50% and 33.3% respectively (table III) (p < 0.01). Treatment with lipase caused no significant reduction.

Discussion

Bovine ruminal and reticular abscesses and rumenitis are frequently observed in slaughterhouses in Japan and F. necrophorum can be isolated from these lesions in heavy and often pure culture (Kanoe et al., 1978, 1984). Adherence of the bacterium to the bovine ruminal mucosa has been recognised as an important early event in infection (Kanoe et al., 1978). Interest has recently focussed on the possible role of bacterial HG in the establishment of infections in man and animals (Atkinson and Trust, 1980; Nagai et al., 1984; Crichton and Walker, 1985). In this study, we observed that F. necrophorum VPI 2891 adhered well to the membrane of bovine ruminal cells. Adherence was inhibited when the bacteria were pretreated with HG antiserum. Furthermore, immunofluorescence studies demonstrated that the purified HG readily became bound to the ruminal cell membranes, but the effect was reduced when the HG was pretreated with HG antiserum. These observations indicate that fusobacterial cell attachment to bovine ruminal epithelial cells is mediated by HG.

Scanning electronmicroscopy revealed that the microvilli on the ruminal cell membrane did not surround the bacterial cells. This contrasted with the interactions between Yersinia pseudotuberculosis and the Hela cell surface (Brunius and Bolin, 1983) and F. necrophorum and the Vero cell membrane (Kanoe et al., 1985). Attempts were also made to determine the nature of the HG-binding site on the ruminal cell membrane by the use of enzymes. A reduction of the adherence activity was brought about by pretreatment with pepsin and
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trypsin, but not lipase. This suggests that the HG-binding site on ruminal cells differs from the Escherichia coli type-1 pili-binding site (Salit and Gotschlich, 1977).

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REFERENCES