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 (Kanoë *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 10^7 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 10^5 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 dried, fixed with methanol 95% v/v for 5 min, washed, and stained with Giemsa's stain. It was then examined by light microscopy ($\times 400$). 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 μ l) 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 μ m. The filtrate was then purified by Sephadex G-100 (Pharmacia, Uppsala, Sweden) column chromatography. The purified HG had a protein content of 40 μ g/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 μ l; 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 (10^5 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; 10^5 cells/ml) was mixed with 2 ml of the pretreatment solution (0.5 μ g/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 χ^2 -square test. A *p*-value of 0.05 or less was reported as significant.

Results

Adherence and haemagglutininability

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 haemagglutininability of *F. necrophorum* and adherence to bovine ruminal cells

<i>F. necrophorum</i> strain	Mean number of adherent bacteria/cell (SD)	Haemagglutination titre
VPI 2891	16.1 (1.2)*	128
S-45	5.0 (0.8)	0
118	7.4 (1.2)	8
Goat	16.5 (1.7)*	64

SD = standard deviation of triplicate determinations.

* = Significantly different (*p* < 0.01) from the lower values.

Fig. 1. Scanning electronmicrograph of bovine ruminal epithelial cells with adherent *F. necrophorum* strain VPI 2891. Bar indicates 5 μ m.

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 rumi-

Table II. Inhibition of adherence of *F. necrophorum* by homologous haemagglutinin antiserum

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

SD = as in table I.

* $\frac{\text{Number of antiserum-treated bacteria/cell}}{\text{Number of untreated bacteria/cell}} \times 100$.

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

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

SD = as in table I.

* $\frac{\text{Number of pretreated bacteria/cell}}{\text{Number of untreated bacteria/cell}} \times 100$.

† 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 μ m.

nal cells (fig. 2a). The fluorescence was greatly reduced when the HG was pre-incubated with specific antiserum for 1 h at 37°C before being used to treat the epithelial cells (fig. 2b).

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 (Kanoë *et al.*, 1978, 1984). Adherence of the bacterium to the bovine ruminal mucosa has been recognised as an important early event in infection (Kanoë *et al.*, 1978). Interest has recently focussed on the possible role of bacterial HG in the establishment of infec-

tions 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 Bölin, 1983) and *F. necrophorum* and the Vero cell membrane (Kanoë *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

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).

We thank Dr S. Tanaka, Faculty of Agriculture, Yamaguchi University for guidance in the techniques of scanning electron-microscopy. This study was supported in part by a grant-in-aid for co-operative research (58360030) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

- Atkinson H M, Trust T J 1980 Hemagglutination properties and adherence ability of *Aeromonas hydrophila*. *Infection and Immunity* **27**:938–946.
- Beachey E H 1981 Bacterial adherence; adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *Journal of Infectious Diseases* **143**:325–345.
- Brunius G, Bölin I 1983 Interaction between *Yersinia pseudotuberculosis* and the HeLa cell surface. *Journal of Medical Microbiology* **16**:245–261.
- Burrows M R, Sellwood R, Gibbons R A 1976 Haemagglutinating and adhesive properties associated with the K99 antigen of bovine strains of *Escherichia coli*. *Journal of General Microbiology* **96**:269–275.
- Crichton P B, Walker J W 1985 Methods for the detection of haemagglutinins in *Aeromonas*. *Journal of Medical Microbiology* **19**:273–277.
- Fader R C, Avots-Avotins A E, Davis C P 1979 Evidence for pili-mediated adherence of *Klebsiella pneumoniae* to rat bladder epithelial cells *in vitro*. *Infection and Immunity* **25**:729–737.
- Kanoe M, Izuchi Y, Toda M 1978 Isolation of *Fusobacterium necrophorum* from bovine ruminal lesions. *Japanese Journal of Veterinary Science* **40**:275–281.
- Kanoe M, Nagai S, Toda M 1985 Adherence of *Fusobacterium necrophorum* to Vero cells. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene A* **260**:100–107.
- Kanoe M, Nouka K, Toda M 1984 Isolation of obligate anaerobic bacteria from bovine abscesses in sites other than the liver. *Journal of Medical Microbiology* **18**:365–369.
- McNeish A S, Turner P, Fleming J, Evans N 1975 Mucosal adherence of human enteropathogenic *Escherichia coli*. *Lancet* **2**:946–948.
- Nagai S, Kanoe M, Toda M 1984 Purification and partial characterization of *Fusobacterium necrophorum* hemagglutinin. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene A* **258**:232–241.
- Salit I E, Gotschlich E C 1977 Type 1 *Escherichia coli* pili: characterization of binding to monkey kidney cells. *Journal of Experimental Medicine* **146**:1182–1194.
- Tajima M, Yagishita T, Nunoya T 1985 Ultrastructure of mycoplasmal capsules as revealed by stabilization with antiserum and staining with ruthenium red. *Japanese Journal of Veterinary Science* **47**:217–223.
- Wilson M H, Collier A M 1976 Ultrastructural study of *Mycoplasma pneumoniae* in organ culture. *Journal of Bacteriology* **125**:332–339.