Surface components of *Bacteroides fragilis* involved in adhesion and haemagglutination

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Summary. The ability of 19 strains of *Bacteroides fragilis* to adhere to buccal epithelial cells (BEC) and to the human intestinal cell line HT-29 Clone 19A, and to agglutinate rabbit erythrocytes was compared. Adhesion to BEC was poor compared with that to the cell line. Adhesion to the latter was high for 21% of the strains, moderate for 37% and poor for 42%. Only 53% of the strains agglutinated rabbit red blood cells and only strain A459 did so strongly. Haemagglutination and adhesion of *B. fragilis* strain A459 were inhibited by sodium periodate, but not by proteases, heat or carbohydrates. These properties were not affected by protease which removed surface appendages. Periodate treatment did not remove the fimbriae or ruthenium red-staining layer, although the capsule was lost. This suggests that carbohydrate residues on the cell surface, possibly forming part of the capsule, are involved in adhesion and haemagglutination by this strain.

Introduction

Bacteroides fragilis is an important anaerobic pathogen that causes wound, intra-abdominal and urogenital infections and bacteraemia.¹ The adhesive properties of *B. fragilis* have been investigated by many workers because they may contribute to its pathogenicity, but the components responsible have not been unequivocally identified. The ability of *B. fragilis* to adhere to intestinal and buccal epithelial cells and to agglutinate erythrocytes has been studied extensively,^{2–8} but there is no agreement as to the identity of the surface components involved. Fimbriae,⁵ capsules^{2,9} and an electron-dense surface layer⁴ have all been implicated as adhesins.

Many enterobacterial haemagglutinins are blocked by specific carbohydrates^{10,11} but haemagglutination by *B. fragilis* is not inhibited by carbohydrates.^{5,6,9} However, it is inhibited by heat, periodate and some enzymes.^{6,7}

We have compared the ability of 19 strains of *B. fragilis* to cause haemagglutination, as shown previously⁸ with their ability to adhere to an intestinal cell line and to buccal epithelial cells (BEC). The nature of the adhesins carried on one of these strains, A459, which is strongly adhesive and haemagglutinating, was investigated in detail.

Materials and methods

Bacterial strains

Bacteroides fragilis strains ATCC 23745 and NCTC 9343 were obtained from the National Collection of Type Cultures. The remaining 17 strains of *B. fragilis* had been isolated from abscesses: strain 2/79 was kindly provided by Dr H. Shah (London Hospital Medical College); strains MRI 2, MRI 3, MRI 6, MRI 7, MRI 8, MRI 9 and MRI 10 were isolated at the Manchester Royal Infirmary; strains A4, A200, A334, A363, A459, A312, SCI 7, SCI 9 and SCI 13 were kindly provided by Dr D. B. Drucker (University of Manchester).

Strains were identified as *B. fragilis* with the API 20A kit (AP/Laboratory Products, Basingstoke) for the identification of anaerobes.

Media and growth conditions

Cultures were grown in an anaerobic cabinet (Don Whitley Scientific, Shipley, W. Yorks; Mark II) at 37°C in an atmosphere of N₂ 80%, H₂ 10%, CO₂ 10% v/v on Columbia Agar (Oxoid CM311) supplemented with horse blood 5% v/v.

Organisms were grown in modified brain heart infusion (BHI) broth, pH 8, containing (/L of distilled water): BHI powder (Oxoid CM225) 37 g, yeast extract 5 g, NaHCO₃ 1 g, KNO₃ 1 g, menadione 500 µg.

Some strains were also grown in FUM medium prepared and sterilised as described by Loesche *et al.*¹² FUM, pH 7.1, contained (/L of distilled water): tryptone 10 g, yeast extract 5 g, glucose 3 g, haemin 2 mg, menadione 1 mg, cysteine hydrochloride 0.5 g, KH_2PO_4 0.45 g, K_2HPO_4 0.45 g, $(\text{NH}_4)_2\text{SO}_4$ 0.9 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.188 g.

Haemagglutination studies

Haemagglutination tests with rabbit erythrocytes were performed as described by Oyston and Handley.⁸ For inhibition studies, strain A459 was washed with PBS (NaCl 8 g/L, K_2HPO_4 1.21 g/L, KH_2PO_4 0.34 g/L, pH 7.4) and resuspended to an OD_{660} of 0.1 (3×10^9 bacteria/ml). The bacterial suspensions were treated with trypsin (Type III, Sigma) and protease from *Streptomyces griseus* (Type XIV, Sigma) at final enzyme concentrations of 2 mg/ml for up to 4 h at 37°C; equal volumes of bacterial suspension and NaIO_4 (100 mM in water) were incubated at 37°C for up to 1 h. After the above treatments bacteria were washed three times in PBS and resuspended to their original volume. Samples were also heated at 85°C for up to 1 h. Mannose and fructose were included in the assay at a final concentration of 1 mg/ml. The treated samples were then used for haemagglutination studies.

Radiolabelling of bacteria

Radiolabelled bacteria were prepared by inoculating 5 ml of FUM containing C^{14} glucose 2 $\mu\text{Ci}/\text{ml}$ from a Columbia blood plate. The cultures were incubated anaerobically for 24 h at 37°C.

Tissue culture

The intestinal cell line HT-29 Clone 19A¹³ was used with the kind permission of Dr C. Labois (Faculte de Medecine Xavier Bichat, Universite Paris VII) and supplied by Dr G. Warhurst (Clinical Pathology, Hope Hospital, Manchester). It was maintained in Nunc tissue-culture flasks in Dulbecco's Modification of Eagle's Medium (Flow Laboratories) with calf serum 10%. The cells were subcultured weekly. When grown in Falcon tissue-culture plates, cell cultures were kept in a humidified atmosphere containing CO_2 5% in air at 37°C.

Adhesion assay

A modification of the assay of Vosbeck and Huber¹⁴ was used to assess the adherence of *B. fragilis* to intestinal epithelial cells. HT-29 tissue-culture monolayers were prepared in Falcon multiwell trays (2 cm² wells) with an inoculum of trypsinised dissociated cells from a confluent culture. Confluent cell layers were washed twice with PBS immediately before use. PBS (0.5 ml) was added to each well. Radiolabelled bacteria were washed three times with PBS and resuspended

to OD_{550} 0.4 (10^9 bacteria/ml). To each of the monolayers was added 0.5 ml of bacterial suspension. Four wells were used for each strain assayed. Trays were shaken on a plate shaker for 150 min at room temperature. Unbound bacteria were removed by washing the wells three times with PBS and the cells were lysed with 1.5 ml of sodium dodecyl sulphate (SDS) 0.5% w/v overnight at room temperature. Samples (0.5 ml) were transferred to Ecoscint A (Mensura Technology Ltd) and the amount of radioactivity was counted by liquid scintillation. The radioactivity bound in the assay was compared with the total count in the inoculum added to the monolayers to give a percentage adherence value. For comparison, a positive control, *Escherichia coli* MA 17 which adhered to the cell line, and a negative control, *E. coli* MA 8 which gave a very low level of adhesion, were included in the assay.

Adhesion inhibition

To ensure that the concentrations of the agents used in the treatments were comparable, with regard to bacterial number, with those used in the haemagglutination inhibition assays, bacterial suspensions of OD_{660} 1.0 were used. However, to keep the adhesion assay conditions constant, suspensions of OD_{550} 0.4 had to be used. Therefore, after treatment, the suspensions were washed and adjusted to OD_{550} 0.4. Since the heat- and carbohydrate-treated samples in the haemagglutination inhibition tests were not washed after treatment, suspensions of OD_{550} 0.4 were used.

Radiolabelled cultures of *B. fragilis* A459 were washed three times with PBS and resuspended to OD_{550} 0.4 or OD_{660} 1.0. The suspension of OD_{660} 1.0 was treated with trypsin, protease and periodate as described for haemagglutination inhibition. The bacteria were then washed three times in PBS and resuspended in buffer to a volume which would give OD_{550} 0.4 for untreated cells. The other suspension (OD_{550} 0.4) was heated at 85°C or tested with the carbohydrates as described above. The treated samples were then assayed for adhesion. Differences between the adherence of treated and untreated suspensions were compared by Student's *t* test.

Effect of mucin on adhesion

B. fragilis A459 and monolayers were prepared and washed as for the adhesion assay. Monolayers were coated with 0.5 ml of a solution of pig gastric mucin (BDH) in distilled water (58 g/L). Excess mucin was removed by gentle washing twice with PBS. The assay was then performed as described above.

Buccal epithelial cell adherence assay

Radiolabelled bacteria were washed with PBS, resuspended to OD_{550} 0.4 (1×10^9 bacteria/ml) and

mixed with BEC in PBS (4×10^5 cells/ml). Bacterial adherence to BEC was assayed by a modification of the method described by Harty *et al.*¹⁵ The radioactivity associated with the BEC was determined by scintillation counting, and the percentage adhesion was calculated as described by Handley *et al.*¹⁶ *Streptococcus salivarius* HB was included as a positive control strain with a high level of adhesion.

Negative staining for fimbriae and fibrils

The effect of trypsin, protease and NaIO_4 on the fimbriae of strain A459 was shown by examining negatively stained preparations of the treated bacteria. Bacteria were washed three times with water and negatively stained as described by Handley and Tipler.¹⁷ A drop of bacterial suspension was placed on a carbon-coated grid (400 mesh, Agar Aids) which had been plasma-glowed in a Nanotech 300S coating unit to produce a hydrophilic surface. The bacteria were then negatively stained with a drop of methylamine tungstate (Emscope) 2%. Grids were examined with a Hitachi 600 electronmicroscope.

Detection of capsules

The effect of NaIO_4 on the capsule of strain A459 was investigated. Capsules were detected by negative staining with Indian ink. One drop of a 24-h bacterial suspension was mixed on a microscope slide with a drop of glucose 10% w/v and a drop of Indian ink. This was spread thinly over the slide, allowed to dry in air, fixed with ethanol and stained with ammonium crystal violet.¹⁸

Ruthenium red fixation

After treatment of strain A459 with sodium periodate, cell surface located acidic polysaccharides were fixed with ruthenium red by the method described by Oyston and Handley.⁸ Sections were examined with a Hitachi 600 electronmicroscope.

Results

Adhesion to BEC was very low for all the *B. fragilis* strains tested—range 1.6–4.8% of cells adhering. These values were low when compared with the positive control *S. salivarius* HB which was highly adherent in the BEC assay (24.8%). In contrast, some strains adhered strongly to the intestinal epithelial cell line (table I). The most adherent strain in the tissue-culture assay was strain A459 (16.5%) and the least adherent was strain A363 (2.2%). The positive control, *E. coli* MA 17, and the negative control, *E. coli* MA 8, gave values of 6.3% and 0.4% respectively. Since a 2-cm² well contains 2×10^5 cells and the number of bacteria/well is 5×10^8 , 16.5% adherence by strain A459 represents approximately 413 bacteria binding/intes-

Table I. Ability of *B. fragilis* to adhere to BEC and intestinal epithelial cells

Strain no.	Percentage of cells with fimbriae*	HA titre*	Percentage adhesion to	
			BEC†	HT-29‡
<i>B. fragilis</i>				
A459	41	++++	3.9(1.4)	16.5(6.8)
A312	35	+	1.6(0.4)	4.7(0.7)
MRI 8	30	—	2.7(0.4)	12.2(3.3)
SCI 7	20	—	3.7(0.2)	9.0(2.2)
MRI 2	19	+++	2.4(0.8)	3.9(0.9)
MRI 10	16	+	3.4(0.4)	9.2(1.1)
2/79	14	—	2.0(0.3)	6.1(0.6)
MRI 9	11	—	4.4(1.0)	4.5(1.4)
MRI 7	11	+	1.6(0.3)	8.8(3.5)
A363	10	—	3.5(0.5)	2.2(0.5)
MRI 3	10	—	4.8(0.8)	3.8(1.2)
SCI 9	8	—	2.7(0.9)	3.8(1.5)
9343	7	—	2.9(1.5)	11.2(1.7)
A200	7	++	2.1(0.2)	5.8(1.1)
MRI 6	6	+	3.4(0.5)	8.3(1.5)
A334	4	+	3.2(0.5)	9.6(1.0)
A4	0	—	3.6(0.7)	2.4(0.6)
SCI 13	0	+	2.7(0.4)	12.1(3.7)
23745	12.3§	+	2.4(0.3)	4.9(2.2)
<i>E. coli</i> MA8	...	...	ND	6.4(1.1)
<i>E. coli</i> MA17	...	...	ND	0.4(0.05)
<i>S. salivarius</i> HB§	...	...	24.8(3.6)	ND

ND, not done.

* See Oyston and Handley.⁸

† BEC values are mean (SD) values from six replicates.

‡ HT-29 adhesion values are mean (SD) values from two separate batches of monolayers, each with four replicates.

§ Strain 23745¹ and *S. salivarius* HB²¹ are fibrillate not fimbriate.

tinal cell. Strains can be divided into poorly adherent strains with less than 125 adhering bacteria/cell, moderately adherent strains with 125–250 bacteria/intestinal cell and highly adherent strains with more than 250 bacteria adhering/cell. By these criteria, 4 (21%) of 19 strains were highly adherent, 7 (37%) were in the medium category and 8 (42%) adhered in low numbers only. Coating the cell monolayer with mucins did not significantly decrease the adhesion of strain A459.

B. fragilis A459 was selected for further study of the surface components responsible for haemagglutination and adhesion. It was highly adherent in the HT-29 assay, had the highest haemagglutination titre, and had a high proportion of fimbriate cells.

Pre-treatment with trypsin or protease, or carbohydrates, or heating to 85°C did not affect the ability of strain A459 to adhere to erythrocytes or the cell line. However, pre-treatment with sodium periodate for 45 min reduced adhesion in the tissue-culture assay by 50% and abolished haemagglutination after 1 h (table II).

Negative staining of the treated samples showed that the fimbriae of strain A459 were partially removed by trypsin and totally removed by protease. Sodium periodate did not reduce the number of fimbriae. Indian ink films revealed that treatment for 1 h with periodate had removed the capsules from the majority

Table II. Effect of treatments on haemagglutination and adhesion of *B. fragilis* A459

Treatment	HA titre	Adhesion score* (%)
Control	32	100
Trypsin 2 mg/ml, 1 h	32	100
Protease 2 mg/ml, 1 h	32	100
NaIO ₄ 100 mM, 15 min	8	ND
30 min	4	ND
45 min	2	ND
60 min	0	50
Heat 85°C, 1 h	32	100
Mannose 1 mg/ml	32	100
Fructose 1 mg/ml	32	100

ND, not done.

* Adhesion score relative to control (100%).

of cells but subsequent ruthenium red staining did not reveal any reduction in the thickness of the ruthenium red staining layer (RRL). Since trypsin-treated and protease-treated cells did not lose their capsules, they were not stained with ruthenium red to detect any reduction in the thickness of the RRL. The results of the structural effects of the treatments on strain A459 are summarised in table III.

Table III. Effect of pre-treatments on the surface components of *B. fragilis* A459

Treatment	Presence of		
	fimbriae* (percentage of cells)	capsule†	RRL‡ (width, nm)
Control	41	+	7.9
Trypsin 2 mg/ml, 1 h	27§	+	ND
Protease 2 mg/ml, 1 h	0	+	ND
Periodate 100 mM, 1 h	35	—	7.7

ND, not done.

* Detected by negative staining with methylamine tungstate 2%; percentage values represent the proportion of fimbriate cells when 100 cells were examined.

† Detected by Indian ink staining.

‡ Mean widths determined by measuring the RRL in 10 cells in thin sections stained with ruthenium red.

§ Fimbriate cells carried fewer fimbriae than did the fimbriate cells of the control.

Discussion

Adhesion by *B. fragilis* strains shows tissue specificity, because adhesion to BEC was very low compared with that to the intestinal cells. This contrasts with work by Pruzzo *et al.*^{5,7} who found that strains which adhered well to an intestinal cell line also adhered well to BEC. Haemagglutination has been taken as an indication of the ability of an organism to adhere to eukaryotic cell membranes¹⁹ but we found no corre-

lation between haemagglutination and adhesion to the intestinal cell line. However, strain A459, which gave the highest haemagglutination titre also adhered in highest numbers to the intestinal cells.

In the gut, epithelial cells are coated with a viscous layer of mucin which prevents colonisation by pathogenic bacteria.²⁰ However, mucin did not significantly decrease adhesion of strain A459 to intestinal cells. Therefore, *in vivo* this organism may either penetrate the mucin layer, adhere to exposed cells where the mucin layer is not continuous, or adhere to the mucin itself.

There has been controversy about which surface components mediate adhesion and haemagglutination in *B. fragilis*. It has variously been suggested that capsules,⁹ the electron-dense layer⁴ and fimbriae⁵ are responsible for adhesion. Some studies have also found a lack of any correlation between *B. fragilis* surface structures and their ability to adhere to erythrocytes^{3,6-8} or intestinal cells.⁷ We found that the fimbriae of strain A459 did not mediate adhesion or haemagglutination because protease treatment removed the structures without affecting either property. However, periodate, which did not remove the fimbriae, was able totally to abolish haemagglutination and also to reduce adhesion to the intestinal cells. That these properties are sensitive to periodate and resistant to proteolytic enzymes is corroborated by Vel *et al.*⁶ Pruzzo *et al.*⁷ found that both adhesion properties were sensitive to Pronase E and to glucosidase. This contrasts with our experience of treating strain A459 with protease, but it agrees with the periodate inhibition we were able to demonstrate.

The fact that adhesion and haemagglutination are sensitive to periodate indicates that carbohydrate residues are important. Treatment with periodate resulted in the loss of the capsule but not the RRL, which may indicate that the capsule has a role in adhesion and haemagglutination. This was suggested by Pruzzo *et al.*⁷ who proposed that both fimbriae and capsules may be involved in haemagglutination and adhesion. Onderdonk *et al.*² found that pre-incubation with capsular material reduced the ability of *B. fragilis* to adhere to rat peritoneal mesothelium. However, Patrick *et al.*⁴ found that, for *B. fragilis* strains, the capsulate cells in the population lacked haemagglutinating ability, and only the non-capsulate subpopulation with an electron-dense surface layer agglutinated erythrocytes. It should also be noted that although the capsule may contribute to adhesion by *B. fragilis* A459, the non-capsulate strain, A334, was able to haemagglutinate and adhere to the cell line. Therefore, carbohydrate residues, such as those that form part of the LPS molecule, the RRL and cell surface glycoproteins could be involved but these possibilities remain to be investigated.

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