BACTERIAL PATHOGENICITY

Binding of extracellular matrix proteins to the surface of anaerobic bacteria

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The binding of fibronectin, vitronectin, collagen and sialoprotein to 65 anaerobic strains was investigated by means of latex agglutination tests. The binding of fibronectin, collagen and lactoferrin to the same strains was also tested by means of ^{125}I -labelled proteins. The strains were isolated from abdominal infections (55%), from the faeces of healthy subjects (29%) or from the depths of tonsils removed at tonsillectomy (16%). The binding of fibronectin and collagen to *Bacteroides fragilis* strains, tested by the latex agglutination assay, was stronger than their binding to other species. The vitronectin binding of the strains was less common, but was always accompanied by fibronectin binding. Binding to fibronectin-coated beads was inhibited by pre-incubation of the bacterial cells with soluble fibronectin and by heat or protease treatment of the bacterial suspension. No inhibition of the binding was observed with carbohydrates. None of the 65 strains exhibited any binding to fetuin or asialofetuin; 8% of the strains had a binding site for mucin. The binding to mucin-coated beads was inhibited by pre-incubation of the cells with mucin. The radiolabelling method indicated a low binding to ^{125}I -fibronectin. The binding of ^{125}I -collagen-I and ^{125}I -lactoferrin was higher for the anaerobic strains tested.

Introduction

Different species of the genus *Bacteroides* are predominant in the human intestinal flora. The species most frequently isolated from faecal samples are *B. vulgatus* and *B. thetaiotaomicron*, whereas *B. fragilis* is the most common anaerobic pathogen in intra-abdominal and pelvic infections [1]. The clinical importance of *B. fragilis* in human anaerobic infections is well known, and the available evidence points to the existence of particular properties in the pathogenicity of this species. Recent studies have demonstrated that the capsule is an important virulence factor in *B. fragilis* strains [2, 3]. Pruzzo *et al.* [4] have shown the ability of pilate *B. fragilis* isolates to agglutinate erythrocytes and to adhere to epithelial and human cells. The adherence of *B. fragilis* and related species to extracellular matrix (ECM) proteins such as fibronectin, vitronectin and laminin has also been proposed as a virulence factor [5, 6].

The process of bacterial adherence depends on a recognition system whereby bacterial surface ligands

bind to a quasi-specific host cell receptor (ECM components) [7]. However, the majority of bacterial pathogens remain localised on the extracellular host surface after attachment.

In recent years, much information has been accumulated about the nature of the ECM constituents. These components can be arranged into four major families: (a) collagens of several different types, (b) elastins, (c) proteoglycans (e.g. heparan sulphate and dermatan sulphate) and (d) structural or connective tissue glycoproteins such as fibronectin, laminin, thrombospondin and vitronectin [8]. Some pathogens colonising various mucosal surfaces bind to sialic acid residues in mucins and to cell surface glycoproteins and glycolipids [9].

Like most other organisms, bacteria require iron for cellular functions. Human and animal hosts provide an environment in which the availability of iron is limited. The majority of iron in man is bound to iron-binding proteins such as lactoferrin and transferrin [10]. Lactoferrin is present in gastrointestinal mucosal surface secretion.

Several bacterial pathogens that colonise epithelial

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cells are known to possess surface-associated haemagglutinins produced under appropriate growth conditions *in vitro*. As erythrocytes and epithelial cells share similar glycoconjugate surface structures, many of these putative tissue cell adhesins can be assayed by simple haemagglutination techniques [11].

The present study examined the binding of different ECM proteins to a collection of anaerobic bacteria originating from various infective processes, or from the stools of healthy individuals. For some strains, haemagglutination was also examined, as were the surface structures of the bacteria by electron microscopy.

Materials and methods

Chemicals

Vitronectin and fibronectin were purified from human plasma by the method of Vuento *et al.* [12]. A collagen preparation (Vitrogen 100^R, containing type-I 95% and type-III 5% collagen, lot no. 87H183) was purchased from Collagen Corporation (Paolo Alto, CA, USA). Collagen type-I (Sigma) was also tested. Pronase E, proteinase K, trypsin, ovalbumin, fetuin, asialofetuin, D-mannose, L-fucose and mucin (Sigma M1778, Lot 112H7025) were purchased from Sigma. Bovine serum albumin (BSA) was obtained from Boehringer Mannheim GmbH. Iodobeads were from Pierce Chemical Company (USA), and latex beads (0.8 µm diameter) from Difco Laboratories. Carboxy-modified latex beads (0.499 µm diameter) were obtained from Seradyn (Indianapolis, USA). Merthiolate was from Kebo Lab. AB, Stockholm, Sweden.

Culture media

Growth conditions may affect the binding of ECM proteins such as fibronectin and collagen-I to bacterial cells [9]. Accordingly, for variation of growth conditions, anaerobic strains were cultured on peptone yeast extract or on brain-heart infusion agar supplemented with horse blood 5%. Brain-heart infusion broth and Wilkins-Chalgren broth were used in parallel. The bacteria were incubated for 48 h in an anaerobic jar at 37°C or at 30°C.

Bacterial strains

The following strains were examined: 21 *B. fragilis* and 15 other *Bacteroides* species isolated from materials obtained at abdominal surgery; 13 strains of *B. fragilis* and six of other *Bacteroides* species from faecal samples from healthy subjects; 10 different anaerobic strains (*Fusobacterium nucleatum* two strains, *Peptostreptococcus prevotii*, three strains and *Prevotella corporis*, two strains) from tonsils removed at tonsillectomy; and three *Clostridium difficile* strains. *Staphylococcus aureus* Cowan I, *S. haemolyticus* E

2498 and *S. haemolyticus* SM 131 were used as positive controls, and *S. saprophyticus* TW 111 and *S. epidermidis* 3380 as negative controls for latex agglutination assays. *Helicobacter pylori* NCTC 11637 was the positive control for the haemagglutination assay and for the particle agglutination assay (PAA) with fetuin- and asialofetuin-coated beads.

Preparation of latex reagents

Latex particles coated with different proteins were prepared according to the method of Naidu *et al.* [9]. Briefly, 1 ml of latex particle suspension (0.8 µm diameter) was mixed with 3 ml of 0.17 M glycine-NaOH buffer (pH 8.2) and centrifuged at 4500 g for 5 min, and the pellets were resuspended in 3 ml of the same buffer. Highly purified protein (100 µg) was added, and the mixtures were kept at 30°C for 12 h on a horizontal shaker at 50 rpm. The mixtures were centrifuged at 9200 g for 5 min at 20°C, and the supernates were discarded. The pellet was resuspended in 2 ml of the glycine buffer containing merthiolate 0.01% and BSA 0.05% and kept at 4°C for 12 h.

The fetuin- and asialofetuin-coated beads were prepared with 100 µl of carboxy-modified latex (0.8 µm) particles (CML), which were washed twice with 0.1 M phosphate buffer (pH 8.1) and suspended and incubated overnight at 4°C in 2 ml of phosphate buffer containing 4 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The suspension was centrifuged (1200 g, 10 min, 4°C), and the particles were suspended in phosphate buffer containing fetuin or asialofetuin 1 mg/ml and incubated overnight at 4°C. The suspensions were centrifuged (12000 g, 10 min, 4°C), and the protein-coated CML particles were washed four times with phosphate buffer and suspended in the same buffer to a final concentration of 1% total beads, and stored at 4°C until used.

Particle agglutination assay

The 65 strains were grown on various media for 48 h. Bacterial colonies were suspended and washed once in 0.02 M potassium phosphate buffer (pH 6.8). Bacterial cells were resuspended in the same buffer to c. OD 1 at 540 nm and immediately tested for PAA [9]. The strains were tested for auto-aggregation by mixing one drop of phosphate buffer and one drop of bacterial cell suspension. Latex beads coated with BSA were also tested as a negative control.

Heat treatment and proteolytic digestion

For enzyme treatment, bacteria were washed twice in PBS, and the cell density was adjusted to OD 1. Proteinase K and pronase E were dissolved (1 mg/ml) in PBS (pH 7.2) and trypsin in PBSA (pH 7.2). Bacterial suspensions and these enzyme solutions were mixed (1:1).

For heat treatment, bacteria were washed twice in PBS, the concentration of bacteria was adjusted to OD 1, and they were incubated in a waterbath at 40°C, 60°C, 80°C or 100°C for 20 min.

Particle agglutination inhibition assay

One hundred µl of inhibitor (fibronectin, 0.1 M D-galactose, D-glucose, L-fucose, N-acetylgalactosamine or mucin) were pre-incubated with an equal volume of bacterial cell suspension for 30 min at 20°C and then mixed with the PAA reagents. Potassium phosphate buffer (pH 6.8) was used as a negative control.

Haemagglutination and haemagglutination inhibition assay

Harvested bacterial cells were suspended in PBS (pH 7.2) to a final colony count of 10⁹ cells/ml. Equal volumes (50 µl) of a bacterial suspension in PBS and a 1% erythrocyte suspension were mixed in the wells of a microtitration plate and allowed to settle for 1–2 h at 20°C. PBS was used as a negative control.

For the haemagglutination inhibition assay, the bacterial cell suspensions were diluted with PBS to give 4 HAU (haemagglutinating units). The solutions of inhibitors were also prepared in PBS (pH 7.2) at concentrations of 1 mg/ml or 2.5 mg/ml. Inhibition tests were performed in the wells of a microtitration plate with erythrocyte 1% suspensions. Bacterial cell suspensions (50 µl) were mixed with 30 µl of inhibitor solution for 1 h; 50 µl of erythrocyte suspension were then added, and the suspension was allowed to settle at room temperature for 1–2 h. Controls contained 30 µl of PBS instead of inhibitor solution.

¹²⁵I-labelled protein binding assay

Fibronectin, collagen-I and lactoferrin were labelled with iodine according to a modified chloramine-T method with Iodobeads [13]. The labelled proteins were kept at 4°C and used within 3 weeks. Radio-labelled proteins were diluted to c. 30 000 cpm/50 µl in PBS (pH 7.2) containing BSA 1% and added to 100 µl of a bacterial cell suspension. After thorough mixing, samples were incubated at room temperature for 1 h. The binding reaction was stopped by the addition of 2 ml of ice-cold PBS containing Tween 20 0.1%.

Supernates were aspirated after centrifugation at 3000 rpm for 15 min and the radioactivity of the pellets was measured in a gamma-counter. For each experiment, negative control test tubes with all ingredients except bacteria were included and the radioactivity was determined. *S. aureus* strain Cowan I was used as a positive control.

Electron microscopy

Selected *B. fragilis* strains were incubated for 48 h in Wilkins-Chalgren anaerobic broth in an anaerobic jar, and on peptone yeast extract agar supplemented with horse blood 5%. The bacteria were washed carefully in 0.02 M phosphate buffer (pH 6.8) and centrifuged at 1600 g for 15 min. For examination of the bacterial cell surface structures by electron microscopy, negative staining with phosphotungstic acid 2% (pH 7.2) for 1 min was used.

Results

Influence of growth conditions on PAA

In total, 65 anaerobic strains (*Bacteroides*, *Prevotella*, *Fusobacterium*, *Peptostreptococcus* and *Clostridium*) were grown on different media at different temperatures and were tested in PAA with latex particles coated with fibronectin or collagen-I (Table 1). Blood agar proved to be the optimal culture medium for anaerobes because only 34% of the strains displayed auto-aggregation and the binding of the strains was stronger than when they were cultured in other media (brain-heart infusion broth and agar, Wilkins-Chalgren broth). Incubation at 30°C or at 37°C made no difference to the binding of the strains.

Binding of fibronectin, collagen-I and vitronectin to anaerobic bacteria in PAA

Fifty-five *Bacteroides* isolates and 10 other anaerobic strains were examined with different proteins immobilised on latex beads (Tables 2 and 3). The binding of fibronectin and collagen-I was stronger to *B. fragilis* strains isolated from infections and from the faeces of healthy individuals than to strains of other *Bacteroides* species (Table 2). No significant differences were observed in the binding of the different proteins to the strains isolated from infections or from healthy

Table 1. Influence of culture medium on fibronectin and collagen binding of different anaerobic strains examined by PAA

Culture medium	Strains giving positive PAA (%) with		Strains exhibiting AA (%)
	Fibronectin	Collagen-I	
PY blood agar	46	55	34
BHI blood agar	32	49	46
BHI broth	29	44	42
Wilkins-Chalgren broth	30	44	43

AA, auto-agglutination; BHI, brain-heart infusion; PY, peptone yeast extract.

Table 2. Binding of *Bacteroides* strains to different ECM proteins investigated by the latex agglutination method

		Number of strains that gave positive PAA reactions with									
		Fibronectin			Collagen-I			Vitronectin			
Species	Number of strains	+++ ++	+	—	+++ ++	+	—	+++ ++	++	—	AA
Abdominal infections											
<i>B. fragilis</i>	21	7	5	0	8	4	0	1	7	5	9
<i>B. ovatus</i>	3	0	2	0	1	1	0	0	1	1	1
<i>B. thetaiotaomicron</i>	1	0	0	1	0	1	0	0	0	1	0
<i>B. multiacidus</i>	1	0	0	1	1	0	0	0	0	1	0
<i>B. vulgatus</i>	1	0	0	0	0	0	0	0	0	0	1
<i>Bacteroides</i> spp.	9	3	1	0	3	1	0	2	0	2	5
Total	36	10	8	2	13	7	0	3	8	10	16
Faecal samples of healthy individuals											
<i>B. fragilis</i>	13	5	3	3	7	2	2	0	3	8	2
<i>B. ovatus</i>	1	1	0	0	1	0	0	0	0	1	0
<i>B. vulgatus</i>	2	0	1	0	0	1	0	0	0	2	0
<i>Bacteroides</i> spp.	2	1	0	1	1	1	0	0	0	2	0
Total	18	7	4	4	9	4	2	0	3	13	2

AA, autoagglutination.

subjects. The binding of vitronectin was less common, but was never observed in the absence of fibronectin binding. Some of the anaerobic strains displayed agglutination with ovalbumin-coated beads, which is the reason why beads coated with BSA were used as a negative control.

Of the 10 other anaerobic strains, only one *C. difficile* strain gave a strong positive PAA reaction with beads coated with fibronectin, collagen-I or vitronectin. Collagen-I bound to a *P. corporis* isolate (Table 3).

Only 10 of the 65 anaerobic strains exhibited agglutination with mucin-coated beads. These were eight strains of *B. fragilis*, one of *P. corporis* and one of *C. difficile*. None of the strains examined bound to fetuin- or asialofetuin-coated beads.

Inhibition of the binding of fibronectin and mucin in the PAA

Fibronectin PAA was completely inhibited by pre-incubation of the bacterial suspension with soluble fibronectin 1 mg/ml. Other glycoproteins or carbo-

hydrates, such as sucralfate, suramin, fragmin, protamine, polylysine, dextran sulphate 500, pentosan sulphate and heparin, did not inhibit the binding of fibronectin to the cells. Pre-incubation of the cells with mucin 1 mg/ml totally abolished the binding of mucin to the cells. Cell surface structures of *B. fragilis* and other anaerobic strains were sensitive to all proteases used (pronase E, proteinase K and trypsin).

Haemagglutination

The ability of strains of *B. fragilis* and other *Bacteroides* spp. to agglutinate sheep and human red blood cells was examined. Twelve strains displayed a haemagglutination ability, whereas 43 strains did not agglutinate the red blood cells tested. All those strains that demonstrated a haemagglutination ability proved to be auto-aggregating strains, so the binding of fetuin and asialofetuin (the sialoproteins present on the erythrocyte membranes) to them could not be examined by the latex agglutination method. Those *Bacteroides* strains that haemagglutinated sheep or human red blood cells were investigated by electron microscopy to find pili responsible for the haemagglutination activity.

Table 3. Binding of different anaerobic strains to ECM proteins investigated by the latex agglutination method

Species	Number of strains	Number of strains that gave positive PAA reactions with									AA
		Fibronectin			Collagen-I			Vitronectin			
		+++ ++	+	—	+++ ++	+	—	+++ ++	+	—	
<i>C. difficile</i>	3	1	0	1	1	0	1	1	1	0	1
<i>P. corporis</i>	2	0	0	1	1	0	0	0	0	1	1
<i>P. prevotii</i>	3	0	0	3	0	3	0	0	0	3	0
<i>F. nucleatum</i>	2	0	0	2	0	0	2	0	0	2	0
Total	10	1	0	7	2	3	3	1	1	6	2

After negative staining, no pilus-like structures were found on any strain tested.

Binding of radiolabelled proteins to anaerobic bacteria

The binding of ^{125}I -labelled fibronectin, ^{125}I -labelled collagen-I and ^{125}I -labelled lactoferrin to all 65 anaerobic strains was tested. Only 29% of the strains bound to ^{125}I -fibronectin. The binding of ^{125}I -collagen-I to the anaerobic strains was similar to that observed in the PAA; 70% of the strains showed a binding of $\geq 10\%$ of the ^{125}I -labelled collagen-I, in parallel with the strong (+++) agglutination in the PAA. The binding of ^{125}I -lactoferrin was extremely high (30–35%) for six *B. fragilis* isolates (Fig. 1).

Discussion

An important early event in the development of many bacterial infections is the adherence of the pathogens to epithelial and endothelial cell surfaces. Connective tissue proteins such as collagen, laminin and serum-derived fibronectin and vitronectin may all be involved in tissue colonisation in surgical and other infections [14]. The binding of fibronectin and collagen to different aerobic bacterial pathogens has been tested fairly frequently [5, 9, 15]. Far fewer data are available on the binding of vitronectin [5, 6, 15]. The binding of different matrix proteins to *S. aureus* and various

coagulase-negative staphylococci has been investigated most frequently, which indicates the important role of these interactions in special infection processes [9, 16]. The binding of ECM proteins to different bacterial cells may have a dual role in host-parasite interactions. The different cell-surface binding components specific for the various ECM proteins may be involved in the colonisation of wounds and other tissue lesions, but they may also aid indigenous flora such as lactobacilli in the vagina and *B. vulgatus* in the colon to establish themselves on mucosal surfaces [5, 17].

In the present study, the strains of different species of anaerobes exhibited different capacities to bind ECM proteins. Similarly as has already been shown for other bacterial species, different culture media and growth conditions influence the expression of cell surface proteins of anaerobic species. The growth of the strains on the surface of peptone yeast extract agar plates supplemented with horse blood provided the best conditions for the expression of surface binding structures by *Bacteroides* strains. *B. fragilis* is considered to be the most frequent pathogen involved in polymicrobial infections after abdominal surgery. The binding of different matrix proteins to *B. fragilis* strains, as demonstrated by means of PAA and the ^{125}I -labelled protein binding assay, suggests that specific binding may be implicated in the adherence of these strains to epithelial surfaces, similar to the processes observed with other pathogens [9, 15, 18–20]. The results of this study indicate that *Bacteroides*

Fig. 1. Binding of anaerobic bacteria to ^{125}I -labelled matrix proteins: $\square$, fibronectin; $\blacktriangle$, collagen-I; $\bullet$, lactoferrin.

strains contain components that recognise fibronectin and collagen type-I (both immobilised and soluble forms), which can all bind effectively to members of the *Bacteroides* genus. Protease treatment of bacterial cells resulted in complete loss of the reactivity to fibronectin-PAA, which indicates the proteinaceous nature of the respective cell surface receptors. All strains which were isolated from infective processes and did not exhibit auto-aggregation adhered to fibronectin and collagen-I in the PAA. Sixty-one and 69% of the *B. fragilis* strains isolated from faeces also had binding components for fibronectin and collagen-I, respectively. On the other hand, *B. vulgatus* strains, much less frequently involved in infective processes, were also found to possess a binding component for fibronectin. This means that the adherence of *Bacteroides* spp. to fibronectin and to other ECM proteins is not necessarily connected with their pathogenicity, but may be the critical step for tissue invasion, especially if the adherence event induces other reactions, possibly leading to the degradation of basement membranes.

Lactoferrin is an iron-binding glycoprotein secreted by glandular epithelial cells and present in the serum and on mucosal surfaces. It has been suggested that lactoferrin, having a high affinity for iron, removes this element from the iron-dependent metabolic processes of certain micro-organisms, thereby causing an antibacterial effect. On the other hand, the capacity of bacteria to bind lactoferrin may be correlated with virulence [21]. This study revealed a high level of binding of ¹²⁵I-lactoferrin to anaerobic bacteria, and especially those *Bacteroides* strains isolated from infective processes or from the faeces of healthy individuals. Further studies are needed to determine whether the lactoferrin binding of *Bacteroides* strains may be of importance in infective processes.

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