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The role of neuraminidase in haemagglutination and adherence to colon WiDr cells by *Bacteroides fragilis*

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Summary. The role of neuraminidase in haemagglutination and adherence to colon WiDr cells by eight strains of *Bacteroides fragilis* and four strains of oral black-pigmented gram-negative anaerobes was studied. Neuraminidase treatment resulted in a very small increase of haemagglutination by some of the strains but had no effect on adherence to WiDr cells by all bacterial strains tested except one strain of *Prevotella intermedia* (HG 110). Inhibition of neuraminidase had no effect on haemagglutination or adherence, nor was any correlation found between haemagglutinating ability and neuraminidase activity in the *B. fragilis* strain. The results indicated that haemagglutination and adherence of *B. fragilis* to WiDr cells were not mediated by neuraminidase.

Introduction

Bacteroides fragilis is a normal inhabitant of the human alimentary tract and plays an important role in infections.[1–3] Neuraminidase (NA), which is found in various pathogenic bacteria, has been thought to contribute to the pathogenicity of *B. fragilis*.[4–7] Bacterial adherence to cell surfaces may involve lectin-like sites on the surface of micro-organisms that bind to specific ligands on the mammalian cells. Neuraminidase specifically cleaves N-acetylneuraminic acid (NANA) from the cell membrane and exposes β-galactosyl determinants which may function as ligands.[8] Okuda *et al.*[9] have found that *Prevotella intermedia* HG 110 does not agglutinate erythrocytes; however, NA treatment of erythrocytes resulted in strong agglutination by *Pr. intermedia*. Guzman *et al.*[10] have suggested a possible role for NA of *B. fragilis* in adherence to both erythrocytes and epithelial cells. The purpose of this study was to investigate the influence of NA on adherence to WiDr cells derived from the colon and on haemagglutination (HA) by strains of *B. fragilis*, *Pr. intermedia* and *Porphyromonas gingivalis*.

Materials and methods

Bacterial strains

Pr. intermedia (formerly *B. intermedius*) strains HG 189 and HG 653 were isolated from patients with periodontitis; *Pr. intermedia* strain HG 110 (ATCC 25611) and *P. gingivalis* (formerly *B. gingivalis*) strain HG 372 (ATCC 33277) were obtained from the American Type Culture Collection. *B. fragilis* strains were isolated from clinical specimens at the Academic Hospital of the Vrije Universiteit, Amsterdam.[11] All strains were grown in BM broth,[12] pH 7·4, supplemented with haemin (BDH) 5 mg/L and menadione (Merck) 2 mg/L at 37°C in an anaerobic chamber (Coy's Manufacturing Co., USA) with an atmosphere of N_2 80%, H_2 10% and CO_2 10% for 48 h.

Haemagglutination (HA) test

Chicken red blood cells (RBC) were washed three times with phosphate-buffered saline (PBS, pH 7·4) and suspended in PBS to give a concentration of 2% v/v. Bacterial cells were grown for 48 h, washed three times, then suspended in PBS to a concentration of $2{\cdot}5 \times 10^9$ cfu/ml, as estimated by optical density. Serial two-fold dilutions of the bacterial suspensions were made in 50 μl volumes of PBS in a round-bottomed microtitration plate and 50 μl of RBC suspension were added to each well. The plate was shaken gently for 1 h and held overnight at 4°C. The HA titre was expressed as the highest dilution of bacteria that showed HA activity. Peanut lectin (PNA) from *Arachis hypogaea* (Sigma) at a concentration of 16 μg/ml was used as a positive control.

Adherence to WiDr cells

A monolayer of WiDr cells (ATCC; CCL 218) was prepared in a 50-ml tissue-culture flask (Costar). The cells were seeded at 5×10^6 cells/flask in DMEM

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(Falcon Laboratories) supplemented with fetal bovine serum (Gibco) 20%, penicillin 100 U/ml and streptomycin 100 μg/ml (Falcon Laboratories) and incubated at 37°C in air with CO_2 10% for 72 h. The cells were washed with PBS and resuspended in 1 ml of DMEM with serum and without antibiotics; 35 μl were then immobilised on a glass coverslip (diameter 15 mm) in a 24-multiwell flat-bottomed tissue-culture plate and incubated overnight at 37°C. Non-adherent cells were washed off and 50 μl of bacterial suspension in PBS (5×10^8 cfu/ml) and 0·5 ml of DMEM without antibiotics were added to the coverslip and incubated at 37°C for 1 h. The cells were then washed with PBS, fixed with acetone, stained with fuchsin and examined by light microscopy at a magnification of × 1000. The numbers of bacterial cells adhering to each of 50 WiDr cells were counted in duplicate. Adhesion was expressed as the mean number of adherent bacteria/cell.

Enzyme and enzyme inhibitor

The enzyme NA from *Vibrio cholerae* and the NA-inhibitor,[13] 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Boehringer Mannheim) were used at concentrations of 0·05 U/ml and 10 mmol/ml respectively. These were the highest concentrations that did not cause lysis of the RBC after incubation at 37°C for 1 h. Treatment with NA or NA-inhibitor, or both, was performed with the RBC 10% suspension and the WiDr cells (5×10^6) at 37°C for 1 h. The cells were washed three times with PBS and the concentration of RBC was adjusted to 2% in PBS. WiDr cells were suspended in PBS to the original volume. Samples containing RBC or WiDr cells without NA or NA-inhibitor were also prepared and used as controls. Bacterial cells ($2{\cdot}5 \times 10^9$ cfu/ml) were also treated with either NA or NA-inhibitor and with both, washed three times then resuspended in PBS to the original volume, before testing for HA and adherence to WiDr cells.

Detection of NA in bacterial cell extract

Bacterial cells were grown in BM broth for 48 h, washed three times in PBS and suspended in PBS to a concentration of 5×10^8 cfu/ml. The washed cells were disrupted for 3 min with an MSE 150 W ultrasonic disintegrator.

Fetuin (Sigma) at a concentration of 5 mg/ml was used as substrate; 0·2 ml of bacterial cell extract was added to 0·2 ml of substrate and incubated at 37°C for 18 h. Inhibition of enzyme activity was investigated by adding 0·1 ml of different concentrations of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid and incubating at 37°C for 18 h. The reaction was stopped by the addition of 0·1 ml of 0·2 M sodium metaperiodate in 9 M *o*-phosphoric acid to 0·1 ml of cell extract and substrate. The enzymatically released NANA was measured in a Gilford spectrophotometer as described previously.[11]

Results

Effect of NA and NA-inhibitor treatment of RBC on HA by bacterial strains

The effect of pre-treatment of RBC with NA, NA-inhibitor and with both, on the ability of bacterial strains to cause HA is shown in table I. Untreated RBC failed to aggregate when exposed to PNA, but after treatment with NA, agglutination took place. NA-inhibitor prevented agglutination of NA-treated RBC by PNA, which confirms that 2-deoxy-2,3-N-acetylneuraminic acid inhibits the activity of NA from *V. cholerae*. All strains apart from those of *Pr. intermedia* agglutinated untreated RBC to varying degrees; NA treatment of RBC did not lead noticeably to improved HA except with *Pr. intermedia* HG 110. *B. fragilis* strains BE 12, BE 49 and BE 61, *Pr. intermedia* strains HG 189 and HG 653, and *P. gingivalis* strain HG 372 showed a two-fold increase in HA when RBC treated with NA were used. *Pr. intermedia* HG 110 showed a 32-fold increase in HA activity with NA-treated RBC. Unfortunately, this strain was auto-agglutinable and its adherence to WiDr cells could not be measured accurately. Treatment of RBC with NA, either with or without NA-inhibitor, did not affect HA when compared to controls (untreated RBC). *B. fragilis* BE 17 showed no HA activity with the bacterial concentrations used in this study but had a high NA activity (32·8 nmol/min/10^8 cfu). On the other hand, *B. fragilis* BE61 showed a higher HA titre (32) but low NA activity (20·0 mmol/min/10^8 cfu). These results suggest strongly that there is no correlation between HA and NA activity in *B. fragilis* strains. Treatment of bacterial strains with NA, its inhibitor, and with both, did not change the HA patterns when compared to the untreated group.

Effect of treatment of WiDr cells with NA and NA-inhibitor on the adherence of bacterial cells

The effect of pre-treatment of WiDr cells with NA and NA-inhibitor on the subsequent adherence of bacterial cells is shown in table II. Bacterial strains varied in their ability to adhere to WiDr cells; *B. fragilis* BE 17, which did not agglutinate RBC, adhered in small numbers to WiDr cells whereas *B. fragilis* BE 61, which had a high HA titre, had the highest number of bacteria attached per WiDr cell. However, no relationship was found between HA and adherence to WiDr cells in the other bacterial species and strains. The treatment of WiDr cells with NA or NA-inhibitor did not affect adherence when compared to untreated cells. The treatment of bacterial strains with NA, its inhibitor and with both, did not affect adherence when compared to untreated bacteria.

Table I. HA titres and NA activity of bacterial strains and peanut lectin (PNA) with RBC treated with NA and NA-inhibitor (NI)

Bacterial strains	HA titres with RBC that were				Mean (SD) NA activity (nmol/min/ 10^8 cfu)*
	untreated	pre-treated with			
		NA	NA+NI	NI	
PNA	0†	64	0	0	—
B. fragilis					
BE 1	16	16	16	16	19·0 (2·2)
BE 2	8	8	8	8	34·0 (3·5)
BE 4	32	32	32	32	22·0 (3·2)
BE 12	8	16	8	8	36·6 (5·6)
BE 17	0‡	0	0	0	32·8 (5·6)
BE 43	32	32	32	32	30·2 (4·3)
BE 49	16	32	16	16	18·0 (4·7)
BE 61	32	64	32	32	20·0 (4·1)
Pr. intermedia					
HG 189	0	2	2	2	ND
HG 653	0	2	2	2	ND
HG 110	0	32	2	2	ND
P. gingivalis					
HG 372	2048	4096	2048	2048	ND

ND, not done.
* All values calculated are the mean (SD) of three experiments.
† No agglutination with 16 μg/ml lectin.
‡ No agglutination with $1{\cdot}2 \times 10^9$ cfu/ml.

Table II. Adherence of bacterial cells to WiDr cells treated with NA and NA-inhibitor (NI)

Bacterial strains	Adherence to WiDr cells* that were		
	untreated	treated with	
		NA	NI
B. fragilis			
BE 1	1·7 (0·10)	1·6 (0·21)	1·8 (0·20)
BE 2	2·2 (0·11)	1·8 (0·20)	2·0 (0·19)
BE 4	1·5 (0·08)	1·7 (0·10)	2·0 (0·06)
BE 12	3·3 (0·14)	3·0 (0·57)	2·4 (0·10)
BE 17	0·4 (0·06)	0·3 (0·04)	0·4 (0·10)
BE 43	2·4 (0·21)	2·4 (0·17)	3·2 (0·35)
BE 49	3·3 (0·42)	2·9 (0·42)	3·4 (0·40)
BE 61	4·5 (0·71)	4·6 (0·35)	4·6 (0·51)
P. intermedia			
HG 189	0·7 (0·40)	0·6 (0·35)	0·7 (0·41)
HG 653	0·6 (0·40)	0·6 (0·30)	0·7 (0·35)
P. gingivalis			
HG 372	1·2 (0·50)	1·6 (0·41)	1·0 (0·48)

* All values are the mean (SD) of three experiments; each value represents the number of attached bacteria/cell.

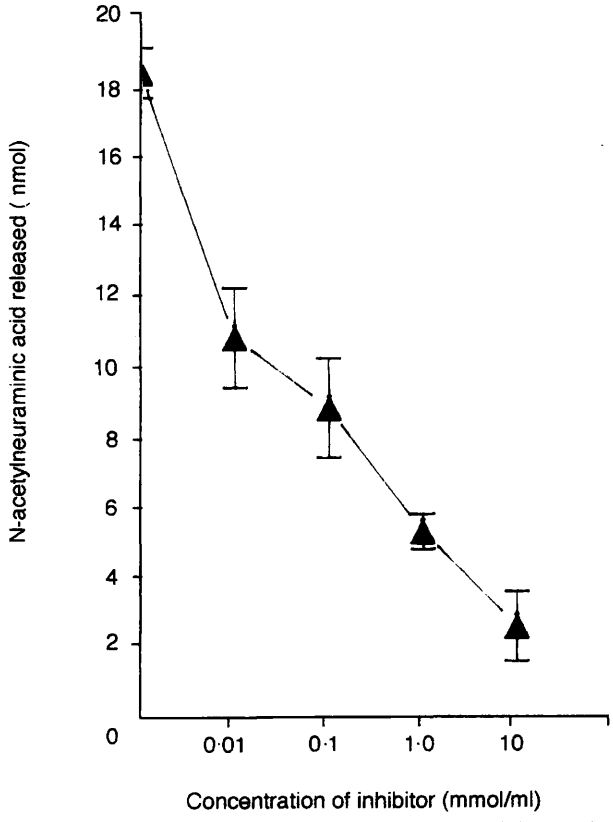

Figure. Effect of 2-deoxy-2,3-dehydro-N-acetylneuraminidase acid, on NA activity of *B. fragilis* BE 61. Values are the mean and SD of three experiments.

Effect of NA-inhibitor on NA activity of B. fragilis

To investigate whether 2-deoxy-2,3-dehydro-N-acetylneuraminic acid could act as a competitive inhibitor of *B. fragilis* neuraminidase activity, different concentrations of NA inhibitor were tested on the NA activity of bacterial cell extract (figure). NA inhibitor at a concentration of 10 mmol/ml inhibited NA activity of *B. fragilis* BE 61 by 85%. These results showed that NA activity of *B. fragilis* is inhibited by the NA-inhibitor.

Discussion

Colonisation of an epithelial surface is a complex process in which adherence is the first step. The affinity of a micro-organism for an epithelial surface is

determined by the outcome of the interaction of the complex mixture of specific and non-specific factors that operate *in situ*. Factors such as the physical barrier of the mucus gel, the electrostatic repulsion between bacterial and epithelial cell surfaces, and competition by receptor analogues in mucosal secretion all interact at mucosal surfaces. It can be argued that the use of RBC and a transformed tissue culture cell line to measure adherence in relation to mucosal surfaces is artificial. However, mammalian cell lines *in vitro* give a relatively uniform population of cells that can be infected under defined conditions. This enables selective modification of either the infective agent or the host cell to be measured.

In the present investigation, the role of NA on HA and adherence of *B. fragilis* strains was studied primarily. Strains of *Pr. intermedia* and *P. gingivalis* were also included because NA-dependent HA and adherence have been reported for *Pr. intermedia*.[9] We preferred to use colon WiDr cells rather than buccal epithelial cells and small intestine cells (I407) because *B. fragilis* is a normal inhabitant of the colon. Treatment of RBC or *B. fragilis* with NA resulted in a very small increase of HA (two-fold) in some of the strains but NA-inhibitor had no effect. The possibility that NA-inhibitor could not act on NA of *B. fragilis* was ruled out by demonstrating that the NA-inhibitor, at concentrations used for HA and adherence studies, inhibited NA activity of *B. fragilis* BE 61 by 85%. Guzman *et al.*[10] found that pre-treatment of RBC with NA increased the ability of *B. fragilis* to adhere to RBC, oral epithelial cells and intestinal cells. However, they did not report the titre of NA-mediated HA and made no attempt to investigate whether NA-mediated HA and adherence to intestinal cells could be inhibited by NA-inhibitor. Further evidence that points to *B. fragilis* HA being independent of NA was the fact that no correlation was found between HA and NA activity. Adherence of *B. fragilis* strains to WiDr cells was not mediated by NA nor was it affected by inhibition of NA. The absence of a relationship between HA and adherence of bacterial strains to WiDr cells agrees with the findings of Oyston and Handley,[14] who used HT-29 colon cells. However, our finding that NA had no noticeable effect (only a two-fold increase) on the HA activity of the *Pr. intermedia* and *P. gingivalis* strains tested contrasts with the work by Okuda *et al.*[9] Moreover, *Pr. intermedia* HG 110, which was also investigated by Okuda *et al.*[9] showed NA-mediated HA, and was auto-agglutinable in our hands, thus effectively rendering adherence assays with WiDr cells impossible. NA-dependent HA of auto-agglutinable strains is an interesting observation which merits further investigation. However, our results indicate that HA and adherence to WiDr cells by *B. fragilis* are not mediated by NA.

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