

Adhesion of *Gardnerella vaginalis* to vaginal epithelial cells: variables affecting adhesion and inhibition by metronidazole

M PEETERS AND P PIOT

From the Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium

SUMMARY Variables affecting the adherence of *Gardnerella vaginalis* to human vaginal epithelial cells were examined in vitro. Adherence depended on pH, with maximum attachment occurring between pH 5 and pH 6. Preincubation of the bacteria at 56°C for 30 minutes and ultraviolet irradiation resulted in a noticeable decrease in adherence. In contrast, adherence was not altered by preincubating the epithelial cells under these conditions. Periodate oxidation of the vaginal cells caused an appreciable reduction in subsequent adherence of *G vaginalis*. None of the 19 single carbohydrates tested inhibited adherence completely.

Metronidazole at subinhibitory concentrations for *G vaginalis*, appreciably reduced the adhesive capacity of *G vaginalis*, whereas subinhibitory concentrations of ampicillin did not.

Introduction

Gardnerella vaginalis and obligate vaginal anaerobes have been implicated in the pathogenesis of bacterial vaginosis (non-specific vaginitis), the main signs of which include an abnormal vaginal discharge, an increased vaginal pH (pH > 4.5), the liberation of characteristic amines on alkalisation of vaginal secretions, and the presence of "clue" cells in vaginal fluid. "Clue" cells are vaginal epithelial cells covered by large numbers of Gram negative to Gram variable coccobacilli.^{1,2}

As is the case in several infections, adhesion of *G vaginalis* to epithelial cells may be an important step in the pathogenesis of bacterial vaginosis. We evaluated variables influencing adhesion of *G vaginalis* to exfoliated vaginal epithelial cells in vitro. The effect of exposure of *G vaginalis* to subinhibitory concentrations of metronidazole and ampicillin on in vitro adherence was also studied.

Patient, materials, and methods

ORGANISMS AND CULTURE CONDITIONS

We used five strains of *G vaginalis* throughout this

study: strain NCTC 10287 (ATCC 10418), and four strains (2301-1, 68931, 68976, and 68977) isolated in our laboratory from patients with signs and symptoms of bacterial vaginosis. The organisms were stored as stock cultures at -70°C in tryptic soy broth (Difco, Detroit, United States of America) with 50% donor horse serum (Gibco, Paisley, Scotland). Strains were cultured on Columbia agar (BBL, Cockeysville, USA) with 5% human blood at 35°C for 48 hours in an atmosphere containing 5% carbon dioxide. Subcultures were made in 2 ml modified PMD broth (proteose peptone no 3 (Difco) 15 g, maltose (Difco) 10 g, dextrose (Difco) 2 g, disodium hydrogen phosphate 0.008 mol/l water (1 g Na₂HPO₄) (Merck, Darmstadt, Germany) and sodium dihydrogen phosphate 0.007 mol/l water (1 g NaH₂PO₄·2H₂O) (Merck) in 900 ml water; supplemented with 20 g yeast extract (Difco) and 100 ml fetal calf serum (Gibco)) incubated for 24 hours at 35°C in an atmosphere of 5% carbon dioxide.

Before performing the adherence assay, we washed the subcultures three times by centrifugation for 15 minutes at 800 × g in phosphate buffered saline (PBS) pH 7.2 (Oxoid, London, England). The final pellet was resuspended in a volume of McIlvaine's citrate-acetate-phosphate buffer pH 5.5. This buffer consisted of two solutions: solution A contained citric acid 0.1 mol/l water (21 g C₆H₈O₇·1H₂O in one litre of water), and solution B contained disodium hydrogen

Address for reprints: Dr P Piot, Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium

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phosphate 0.2 mol/l water (35.6 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in one litre of water). Both solutions had 9 g sodium chloride added.

VAGINAL EPITHELIAL CELLS

Throughout the study we used cells from the same donor, who used oral contraception but did not receive any antimicrobial treatment. We obtained the cells by gently scraping the vaginal mucosa with a sterile cotton swab, which was immediately immersed in PBS. The cell suspension was then washed three times by centrifugation for 10 minutes at $150 \times g$ in PBS to remove all vaginal bacterial flora. Finally we resuspended the cells in McIlvaine's buffer pH 5.5, and counted them in a Neubauer counting chamber.

ADHERENCE ASSAY

We measured bacterial adherence by a modification of the methods of several workers.³⁻⁶ Samples of 1 ml of standard bacterial suspensions (2.1×10^8 bacteria/ml) were mixed with equal volumes of vaginal cell suspensions (10^5 cells/ml) in small, flat bottomed tubes. The ratio of bacteria to vaginal cells was based on preliminary experiments, which had shown that the enumeration of adherent bacteria was most reproducible at a ratio of $10^8:10^5$. The tubes were rotated at 35 rpm at 37°C for 45 minutes in a shaking waterbath.

After the epithelial cells had been incubated, they were washed free of non-adherent bacteria by filtration through a $12 \mu\text{m}$ polyester filter (Nucleopore) held in a millipore filter holder mounted on the end of a syringe. Each filter was washed with 30 ml PBS. To transfer the cells to slides, the filter was carefully removed from the filter holder and inverted into a drop of PBS on a clean microscope slide. The filters were lifted off the slide after about two minutes. The slides were air dried, fixed in methanol for five minutes, and Gram stained. The number of bacteria adherent to epithelial cells was counted under a light microscope with a $\times 1000$ objective. In each experiment 50 epithelial cells were counted. All experiments were performed at least twice.

VARIABLES INFLUENCING THE ADHERENCE OF *G VAGINALIS*

We studied the effect of pH by resuspending the strains of *G vaginalis* and the vaginal epithelial cells in a McIlvaine's citrate-acetate-phosphate buffer at a pH range of pH 3 to pH 8.

Pretreatment of vaginal cells included incubating the cell suspension at 56°C for 30 minutes and ultraviolet irradiation by exposing the cell suspension in an open Petri dish to an ultraviolet lamp for 30 minutes. The distance between the lamp and the surface of the cell suspension was 10 cm, and the irradiation at the surface was $1800 \mu\text{W}/\text{cm}^2$. Cell suspensions were also

treated with 10 mg/l sodium-meta-periodate (Merck) for five minutes at room temperature in 5 ml PBS. The cells were then washed twice in PBS before being resuspended in McIlvaine's buffer to the required concentration.

Pretreatment of bacteria consisted of temperature, ultraviolet, and periodate treatments as described for the epithelial cells. Pretreatment of bacteria with various sugars was by incubating washed bacteria with the respective carbohydrates at concentrations of 0.1 or 0.2 mol/l PBS. Bacteria were washed before the adherence assay was performed.

We tested the effect of antibiotics on adhesion as follows. We measured minimum inhibitory concentrations (MIC) of metronidazole (Specia NV, Brussels) and ampicillin (Beecham, Brussels) for each bacterial strain by using a macrobroth dilution method. Serial dilutions of either compound were prepared in 2 ml PMB broth supplemented with 2% yeast extract and 10% fetal calf serum. The tubes were inoculated with 10^6 bacteria/ml and incubated for 24 hours at 35°C in an atmosphere of 5% carbon dioxide. The MIC was defined as the lowest concentration of antibiotic that inhibited visible bacterial growth. We performed adherence assays with strains of *G vaginalis* grown in the presence of metronidazole or ampicillin at concentrations corresponding to one half, one quarter, or one eighth of the MIC of each antimicrobial.

All experiments included a control, which consisted of an adherence assay of untreated epithelial cells and bacteria performed at the same time.

STATISTICAL METHODS

We obtained the variation of the distribution by calculating the standard error of the mean. The significance of differences between means was analysed using the Kruskal-Wallis test, as the data were not normally distributed and a transformation did not bring the differences between the variances to an acceptable level.⁷

Results

INFLUENCE OF pH ON ADHERENCE

Adherence of *G vaginalis* was maximum at pH 5 to 6 and declined at pH values below and above this range (fig 1). Based on these results, we performed all adherence studies at pH 5.5. The number of adherent bacteria at these conditions varied between 8.4 and $17.4/\text{cell}$.

PRETREATMENT OF BACTERIA AND EPITHELIAL CELLS

Pretreatment of *G vaginalis* with mild heat and ultraviolet irradiation caused a significant ($p < 0.005$) drop in the attachment of the organism to the vaginal

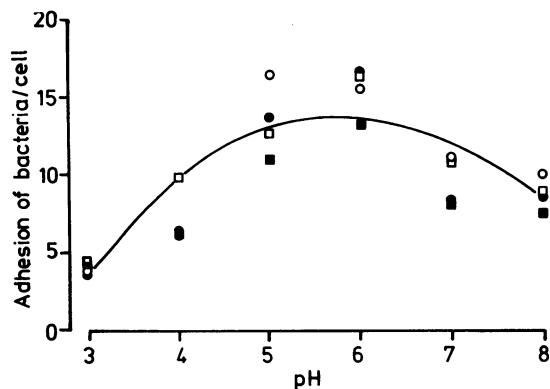


FIG 1 Influence of pH on adhesion of four strains of *Gardnerella vaginalis* (● = strain 68631, ◆ = strain 68976, □ = strain 68977, ○ = strain NTCC 10287) to vaginal epithelial cells. Values shown are means obtained from two experiments. The best curve was fitted through these values with a correlation coefficient of 0.76.

glucopyranoside, N-acetyl-glucosamine, and xylose had no influence on adherence of the bacteria to the vaginal cells. All other sugars tested reduced the attachment rate, but none inhibited adhesion completely (table III). Noticeable and consistent differences were observed between the different strains tested.

EFFECT OF SUBINHIBITORY CONCENTRATIONS OF METRONIDAZOLE AND AMPICILLIN

MICs of metronidazole and ampicillin were 16 mg/l and 0.015 mg/l for strain 2301-1, 16 mg/l and 0.031 mg/l for strains 6893 and 68977, and 4 mg/l and 0.015 mg/l for strain 68976. *G. vaginalis* strains grown in the presence of metronidazole exhibited a greatly altered capacity for adhesion. Thus metronidazole at subinhibitory concentrations caused a noticeable reduction in the adhesion of strains 2301-1, 68976, and 68977, but did not influence the adhesion of strain 68931 to vaginal epithelial cells (fig 2). In contrast,

TABLE I Effect of pretreatment of *Gardnerella vaginalis* on its adherence to vaginal epithelial cells

Treatment	Mean* (SEM) adherence of strain:					p value†
	2301-1	68931	68976	68977	NTCC10287	
Heat (30 min at 56°C)	17 (4)	24 (5)	28 (7)	31 (7)	44 (9)	<0.005
Ultraviolet irradiation	28 (5)	23 (10)	16 (4)	27 (7)	23 (10)	<0.005
Sodium-meta-periodate	61 (13)	97 (2A)	64 (11)	84 (22)	153 (19)	<0.01

* Mean bacteria/cell as percentage of untreated control.

† Kruskal-Wallis test.

TABLE II Effect of pretreatment of vaginal epithelial cells on adherence of *Gardnerella vaginalis*

Treatment	Mean* (SEM) adherence of strain:				
	2301-1	68931	68976	68977	NTCC10287
Heat (30 min at 56°C)	59 (21)	31 (14)	135 (28)	78 (17)	91 (1)
Ultraviolet irradiation	50 (18)	99 (18)	81 (16)	32 (9)	103 (14)
Sodium-meta-periodate‡	22 (5)	27 (7)	32 (6)	27 (8)	33 (8)

* Mean bacteria/cell as percentage of untreated control.

† p < 0.005, Kruskal-Wallis test.

cells (table I). Periodate treatment of the bacteria significantly ($p < 0.01$) reduced the adhesion, but to a lower extent than when the epithelial cells underwent periodate pretreatment ($p < 0.005$). Moreover, only two bacterial strains out of five tested showed a pronounced decrease in adhesion after periodate treatment (table II). Mild heat treatment, for 30 minutes at 56°C, and ultraviolet irradiation of the epithelial cells caused no significant decrease in the attachment of *G. vaginalis* to the vaginal cells.

PRETREATMENT WITH VARIOUS SUGARS

Of all the sugars tested, arabinose, cellobiose, fructose, galactose, mannose, melibiose, α -methyl-

TABLE III Effect of pretreatment of *Gardnerella vaginalis* with selected sugars on adherence to vaginal epithelial cells

Carbohydrate	Mean* (SEM) attachment of strain:				
	2301-1	68931	68976	68977	NTCC10287
Fucose	71 (15)	61 (14)	78 (17)	58 (11)	102 (17)
Glucose	27 (7)	42 (9)	38 (9)	43 (14)	61 (12)
Glycogen	84 (25)	35 (15)	26 (12)	26 (8)	42 (12)
Maltose	40 (8)	45 (9)	56 (12)	54 (15)	80 (13)
Mannitol	74 (23)	84 (24)	53 (12)	67 (19)	49 (10)
N-acetyl-galactosamine	25 (7)	92 (22)	54 (12)	55 (9)	61 (9)
Raffinose	58 (16)	52 (12)	61 (13)	48 (15)	NT (3)
Sucrose	34 (11)	78 (19)	46 (8)	61 (15)	70 (10)
Starch	49 (20)	43 (16)	25 (10)	33 (11)	77 (15)

* Mean bacteria/cell as percentage of untreated control.

NT = Not tested.

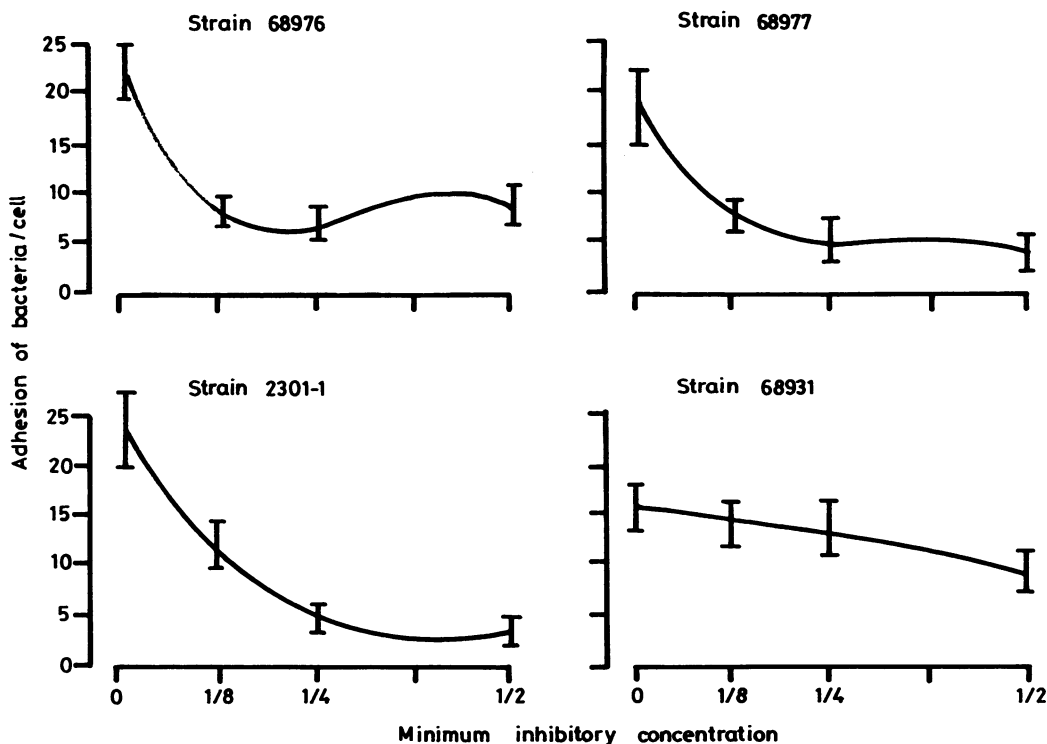


FIG 2 Influence of subinhibitory concentrations of metronidazole on the adherence of four strains of *Gardnerella vaginalis* to vaginal epithelial cells. Values shown are means (bars SEM) obtained from two experiments. The best curves were fitted through these values with correlation coefficients of: 0.95 (strain 2301-1), 0.98 (strain 68931), 0.99 (strain 68976), and 0.97 (strain 68977).

ampicillin at subinhibitory concentrations did not alter the capacity of adhesion of the four strains of *G vaginalis* (data not shown).

Discussion

The study reported here confirms that *G vaginalis* has a good capacity to adhere to vaginal epithelial cells in an experimental model.⁶⁸ The optimum pH for adhesion in vitro was pH 5 to 6 (the vaginal pH of women with bacterial vaginosis), and adhesion was limited at pH 3 to 4 (the pH of vaginal fluid in women without vaginosis). If the same is true in vivo, a rise in vaginal pH is possibly a prerequisite in the pathogenesis of bacterial vaginosis and perhaps precedes the formation of the pathognomonic "clue" cells, which are vaginal epithelial cells covered with *G vaginalis*.

Mild heat treatment and ultraviolet irradiation of the vaginal epithelial cells did not alter the degree of attachment. Treatment with sodium-meta-periodate, which destroys the C-C bond between vicinal hydroxyl groups of carbohydrates, caused an appreciable drop in the number of bacteria attached to

the cells. This suggests that a carbohydrate on the surface of the epithelial cells plays a part in the binding mechanism. We were unable to identify this carbohydrate, however, as attachment to the vaginal cells was not completely inhibited by a single monosaccharide tested. Adherence studies in the presence of the hapten sugars may yield more. A disaccharide, trisaccharide, or larger unit is possibly concerned, and distinct linkages are perhaps necessary to serve as a receptor molecule.^{5-9,13}

On the other hand, mild heat, ultraviolet irradiation, and periodate treatment of the bacteria reduced the degree of attachment. This suggests that a protein, and perhaps a carbohydrate as well, is important for adhesion. This is in contrast with the findings with other bacteria by most workers, in which only a protein was implicated in the function of an adhesin.^{5,12}

Subinhibitory concentrations of various antibiotics have recently been shown to interfere with the capacity of bacteria to adhere to cells.^{14,15} Attachment of *G vaginalis* to vaginal epithelial cells was not influenced by growth of the organism in the presence of subinhibitory concentrations of ampicillin. This contrasts with the influence of ampicillin on the

adhesion of streptococci to buccal epithelial cells,¹² which suggests that streptococci and *G vaginalis* use different adhesion mechanisms or receptors. Subinhibitory concentrations of metronidazole did appreciably reduce the adhesion of *G vaginalis* to vaginal epithelial cells, with a noticeable drop in adherence starting at a concentration equalling one eighth of the MIC of metronidazole. The data also suggest, however, that this may not be the case for all strains of *G vaginalis*, as shown by the results for strain 68931. The mechanism of activity of metronidazole for the treatment of bacterial vaginosis has been a controversial issue.¹⁶⁻¹⁹ Whereas metronidazole has excellent in vitro activity against the anaerobes associated with bacterial vaginosis, it is only moderately active against *G vaginalis*, with MICs ranging between 4 mg/l and 32 mg/l.^{20,21} Our data suggest that part of the activity of metronidazole in bacterial vaginosis is possibly due to a reduction of attachment of *G vaginalis* to epithelial cells. This attachment in itself may be a cause of epithelial cell lysis, and play a part in the pathogenesis of bacterial vaginosis.

Further studies are required to define better the role of adherence of *G vaginalis* to epithelial cells in the pathogenesis of bacterial vaginosis, and to identify both receptors and adhesins concerned in attachment.

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