Chemotaxis inhibition by *Gardnerella vaginalis* and succinate producing vaginal anaerobes: composition of vaginal discharge associated with *G vaginalis*

A W STURM

From the Department of Medical Microbiology, St Laurentius Hospital, Roermond, The Netherlands

SUMMARY The influence of six succinate producing vaginal anaerobes and *Gardnerella vaginalis* on the chemotactic activity of granulocytes was studied by the under agarose method. *G vaginalis*, *Mobiluncus* species, and three Gram negative anaerobes elicited hardly any response, but *Peptostreptococcus productus* showed clear positive chemotaxis, as did the *Escherichia coli* strain used as a control. Inhibition of the chemotactic response of white blood cells was found with all strains, but the high succinate producers from the genus *Bacteroides* showed the most pronounced effect. The inhibition of chemotaxis by succinate producing anaerobes in the pathogenesis of non-specific vaginitis (NSV) is postulated, and *B ureolyticus* or *Mobiluncus* spp, rather than *G vaginalis*, are suggested as possible causes of NSV.

Although the relation between *Gardnerella vaginalis* and abnormal vaginal secretion was described more than 30 years ago,¹ the role of this bacterial species in the clinical condition known as non-specific vaginitis (NSV), bacterial vaginosis, anaerobic vaginosis, Gardnerella associated vaginosis, or clue cell positive vaginal discharge is still not certain.² Most authors have abandoned the word "vaginitis" because of the absence of appreciable numbers of granulocytes in the vaginal contents.³ Because treatment with metronidazole gives good results in this syndrome, anaerobes are thought to play an important if not a major part in its pathogenesis,³ and this is supported by the high succinate concentrations found in the discharge.⁴ *G vaginalis* does not produce this fatty acid.⁵ Several authors have shown that short chain fatty acids interfere with granulocyte function.⁶⁻⁸ The paucity of granulocytes in the vaginal contents of patients with non-specific vaginal disease associated with *Gardnerella*, rather than showing no inflammatory response, is thus possibly the result of short chain fatty acids produced by one or more anaerobes.

In this report I describe the effects of *G vaginalis* and anaerobes of the genital tract on the chemotaxis of granulocytes.

Address for reprints: Dr A W Sturm, Department of Medical Microbiology, St Laurentius Hospital, POB 920, 6040 AX Roermond, The Netherlands

Accepted for publication 19 November 1988

Materials and methods

BACTERIAL STRAINS

Escherichia coli, *G vaginalis*, *Mobiluncus curtisii*, and *M mulieris* were recent clinical isolates from routine vaginal discharge cultures. *Bacteroides ureolyticus* (NGUAA (non-gonococcal urethritis associated anaerobes)) was kindly supplied by Dr Taylor-Robinson of the MRC Clinical Research Centre, Harrow, and the remaining strains were obtained from the ATCC collection; *B asaccharolyticus* (ATCC 25260), *B bivius* (ATCC 29303), and *Peptostreptococcus productus* (ATCC 27340).

PREPARATION OF WHITE BLOOD CELLS

Human white blood cells were prepared as described by Nelson *et al*⁹ with slight modifications. A volume of 20 ml heparinised venous blood was taken from a healthy male volunteer who had not received antimicrobial chemotherapy or other medication within the previous three months. The same donor was used throughout the study, and the chemotactic response of cells from each donation was measured with *E coli* culture filtrate as attractant. Erythrocytes were sedimented by gravity at 37°C. The supernatant fluid that contained leucocytes and a few erythrocytes was collected, and the cells were recovered by centrifugation and washed twice in Hanks' balanced salt solution (HBSS), pH 7.2, containing 0.1% gelatin. The pellet was suspended in 20 ml 0.84% ammonium

chloride and incubated in a waterbath at 37°C for 10 minutes to lyse the residual erythrocytes. The cells were then washed free of the ammonium chloride with HBSS, after which their viability was estimated by trypan blue exclusion. The cells were counted in a haemocytometer and resuspended in HBSS to give a concentration of 10^7 white blood cells/ml.

CHEMOTAXIS ASSAY

Chemotaxis was measured under agarose by the slightly modified method of Nelson *et al.*⁹ Briefly, 5 ml 1% agarose (BDH Chemicals Ltd, Poole, England) in HBSS, supplemented with 10% heat inactivated human serum and 0.375 mg/ml sodium bicarbonate, were delivered to each 60 × 15 mm tissue culture dish (Falcon, California, USA) and allowed to harden. After 60 minutes in the refrigerator, six series of three wells 2.4 mm in diameter and spaced 2.4 mm from each other were cut in each plate using a stainless steel punch. The centre well (figure) of each series received 10 µl white blood cell suspension, representing 10^4 cells. The outer well received 10 µl of a culture of one of the bacterial species that had been cultured for 72 hours in peptone yeast glucose (PYG) broth and diluted 1:2¹⁰ or culture filtrate from which bacteria had been removed by filtration (through a 0.2 µm filter; Millipore, Bedford, USA). The inner well received 10 µl of sterile PYG from the same batch that had been incubated under the same conditions, but without bacteria. The completed dishes were incubated for two hours at 37°C in a humidified atmosphere containing 7% carbon dioxide in air. The migration patterns were measured with an ocular micrometer at 100 times magnification using reversed microscopy. The chemotactic index was calculated as the quotient of the

Chemotactic index = B/A

- 1 Inner well containing sterile peptone yeast glucose broth
- 2 Centre well containing 10^4 white blood cells
- 3 Outer well containing bacteria culture or culture filtrate

Figure Chemotaxis under agarose (chemotactic index = B/A).

linear distance the cells moved from the margin of the well towards the bacterial culture or filtrate in relation to the distance they moved towards the control medium (figure).

INHIBITION OF CHEMOTAXIS

Inhibition of chemotaxis was measured by comparing the chemotactic index of a culture of each species diluted 1:2 with the chemotactic index of the same strain mixed with an equal volume of a *G vaginalis* or *E coli* culture. All experiments were performed 12 times in four series with each combination in triplicate.

SUCCINATE PRODUCTION

Gas liquid chromatography was performed as described by Holdeman *et al.*¹⁰

STATISTICS

Statistical analysis was performed by Fisher's test of exact probability and Student's *t* test.

Results

The differences between responses of PMNL from different blood donations with *E coli* as chemoattractant were not significant. *B ureolyticus* and *B bivius* produced more, but all four other anaerobes produced less than 1 milliequivalent/100 ml succinic acid. No succinic acid production could be detected using *G vaginalis*.

Table 1 shows the results of the chemotaxis experiment with the eight strains tested. Clear positive chemotaxis was found with *E coli* and *P productus*, but *G vaginalis*, both species from the genus *Mobiluncus*, and the three Gram negative anaerobes showed hardly any response of the white blood cells. The same results were found with bacteria free culture filtrates of all eight species tested (data not shown).

Table 2 summarises the results of the chemotaxis inhibition tests. Inhibition with *G vaginalis* as a chemoattractant was significant for *M mulieris* ($p < 0.001$) and *B ureolyticus* ($p < 0.005$). When *E coli* was

Table 1 Chemotactic index of *Escherichia coli*, *Gardnerella vaginalis*, and six genital tract anaerobes in 12 experiments

Bacterial strain	Chemotactic index		
	Mean (SD)	Minimum	Maximum
<i>E coli</i>	2.3 (0.19)	1.9	2.5
<i>G vaginalis</i>	1.3 (0.17)	0.9	1.5
<i>Mobiluncus curtisii</i>	1.2 (0.03)	1.0	1.3
<i>M mulieris</i>	1.1 (0.14)	0.9	1.3
<i>Bacteroides ureolyticus</i>	1.2 (0.14)	1.0	1.4
<i>B bivius</i>	1.2 (0.09)	1.0	1.3
<i>B asaccharolyticus</i>	1.1 (0.11)	0.9	1.3
<i>Peptostreptococcus productus</i>	2.0 (0.19)	1.7	2.4

Table 2 Inhibition of chemotactic response to *Gardnerella vaginalis* and *Escherichia coli* by vaginal anaerobes

	Chemotactic index	Difference between mixture and pure culture
	Mean (SD)	
<i>G vaginalis</i> (pure culture)	1.3 (0.17)	
<i>G vaginalis</i> mixed with:		
<i>Mobiluncus curtisii</i>	1.2 (0.04)	NS
<i>M mulieris</i>	1.0 (0.09)	p < 0.001
<i>Bacteroides ureolyticus</i>	1.1 (0.11)	p < 0.005
<i>B bivius</i>	1.2 (0.10)	NS
<i>B asaccharolyticus</i>	1.3 (0.03)	NS
<i>Peptostreptococcus productus</i>	2.0 (0.14)	NS
<i>E coli</i> (pure culture)	2.3 (0.19)	
<i>E coli</i> mixed with:		
<i>G vaginalis</i>	2.1 (0.12)	p < 0.01
<i>M curtisii</i>	2.5 (0.14)	p < 0.01
<i>M mulieris</i>	2.4 (0.13)	p < 0.01
<i>B ureolyticus</i>	1.4 (0.07)	p < 0.001
<i>B bivius</i>	1.5 (0.09)	p < 0.001
<i>B asaccharolyticus</i>	1.9 (0.11)	p < 0.001
<i>P productus</i>	2.1 (0.12)	p < 0.01

Table 3 Comparison of mean chemotactic index of *Gardnerella vaginalis* and *Escherichia coli* mixed with cultures of vaginal anaerobes and their culture filtrate

	Mean (SD) chemotactic index		
	Whole culture	Culture filtrate	Difference
<i>G vaginalis</i> mixed with:			
<i>Mobiluncus curtisii</i>	1.2 (0.04)	1.2 (0.03)	NS
<i>M mulieris</i>	1.0 (0.09)	1.0 (0.06)	NS
<i>Bacteroides ureolyticus</i>	1.1 (0.11)	1.1 (0.09)	NS
<i>B bivius</i>	1.2 (0.10)	1.2 (0.09)	NS
<i>B asaccharolyticus</i>	1.3 (0.03)	1.1 (0.05)	p < 0.001
<i>Peptostreptococcus productus</i>	2.0 (0.14)	2.1 (0.11)	NS
<i>E coli</i> mixed with:			
<i>G vaginalis</i>	2.1 (0.12)	2.1 (0.10)	NS
<i>M curtisii</i>	2.5 (0.14)	2.4 (0.09)	NS
<i>M mulieris</i>	2.4 (0.09)	2.4 (0.10)	NS
<i>B ureolyticus</i>	1.4 (0.07)	1.4 (0.05)	NS
<i>B bivius</i>	1.5 (0.09)	1.5 (0.11)	NS
<i>B asaccharolyticus</i>	1.9 (0.11)	1.8 (0.08)	p < 0.02
<i>P productus</i>	2.1 (0.12)	2.2 (0.11)	NS

the chemoattractant the most pronounced differences ($p < 0.001$) were found for the three strains from the genus *Bacteroides*, but the other four species were also inhibited significantly ($p < 0.01$). Table 3 gives the results of the inhibition experiments with whole cultures compared with culture filtrates. Only *B asaccharolyticus* showed significant differences ($p < 0.001$) when the chemoattractant was *G vaginalis*, $p < 0.02$ when it was *E coli*.

Discussion

The results of this study show that vaginal anaerobes

are able to inhibit chemotaxis of white blood cells. The influence of variation of white blood cells from different blood donations was ruled out by using the same donor, testing each sample against the same strain of *E coli*, and performing the inhibition experiments in clusters in which all combinations were tested. Except in the case of one strain, the inhibiting substance was shown to be part of the culture filtrate and was probably identical with one or more short chain fatty acid.

Other investigators have reported inhibition of chemotaxis by these acids, of which succinate seems to have the strongest effect.⁸ In the study published here the greatest inhibition was found with the strongest succinate producers, and species that produced less of this acid yielded a much less pronounced effect or showed no inhibition at all. The strong inhibition by *B asaccharolyticus*, which does not produce large amounts of short chain fatty acids, was probably due to a cell related factor.

Vaginal discharge from patients with NSV has an increased succinate concentration, which in the light of our results provides an explanation for the paucity of granulocytes. Ison *et al* also found a high ratio of succinate to lactate, and found *G vaginalis*, anaerobes, and a raised pH in the discharges of patients with trichomoniasis and gonorrhoea, but granulocytes were also present.⁴ Ison *et al* suggested that the diagnostic criteria for NSV, as defined by Spiegel *et al* (a pH of more than 4.5, a typical homogeneous discharge, clue cells, and the release of amines by potassium hydroxide),¹¹ are the results of a primary stimulus that alters the normal ecology of the vagina and results in a raised pH and increased growth of *G vaginalis* and anaerobes.⁴ In infections such as gonorrhoea and trichomoniasis the primary stimulus possibly evokes such a strong chemotactic response that the subsequent inhibition by an increase in the numbers of anaerobes producing short chain fatty acids is too weak to counteract the response. If, however, one of the anaerobes with strong inhibitory potential (such as one of the species from the genus *Bacteroides*) causes the primary process, no positive chemotaxis precedes the inhibiting action of the short chain fatty acids, which results in few pus cells in the discharge.

The results of this study at least throw doubts on the role of *G vaginalis* as an aetiological agent of NSV and suggest that a high succinate producer, such as one of the *Bacteroides* species, could play a part. Because of its possible role in the aetiology of non-gonococcal urethritis¹² *B ureolyticus* is one candidate.

Succinate production from glycogen by *Mobiluncus* species has been reported.¹³ Because experiments in the study published here used bacteria grown in glucose broth, *Mobiluncus* spp may still play a part in the pathogenesis of NSV.

Gardnerella associated vaginal discharge possibly results from infection that does not show all the characteristics of inflammation, which accords with the excellent results of systemic treatment with metronidazole. Histological and bacteriological examination of tissue from the vaginal wall and the cervical canal is needed to explore this hypothesis.

References

- 1 Gardner HL, Dukes CD. New etiologic agent in nonspecific bacterial vaginitis. *Science* 1954;120:853-5.
- 2 van der Meijden WI. Clinical aspects of *Gardnerella vaginalis*-associated vaginitis. A review of the literature. *Scand J Urol Nephrol [Suppl]* 1985;86:135-41.
- 3 Spiegel CA, Davick P, Totten PA, et al. *Gardnerella vaginalis* and anaerobic bacteria in the etiology of bacterial (nonspecific) vaginosis. *Scand J Infect Dis [Suppl]* 1983;40:41-6.
- 4 Ison C, Easmon CSF, Dawson SG, Southerton G, Harris JWR. Non-volatile fatty acids in the diagnosis of non-specific vaginitis. *J Clin Pathol* 1983;36:1367-70.
- 5 Moss CW, Dunkelberg WE. Volatile and cellular fatty acids of *Haemophilus vaginalis*. *J Bacteriol* 1969;100:544-6.
- 6 Botta GA, Eftimiadi C, Costa A, Tonetti M, van Steenberg TJM, de Graaff J. Influence of volatile fatty acids on human granulocyte chemotaxis. *FEMS Microbiology Reviews* 1985;27:69-72.
- 7 Rotstein OD, Pruett TL, Fiegel VD, Nelson RD, Simmons RL. Succinic acid, a metabolic by-product of *Bacteroides* species, inhibits polymorphonuclear leukocyte function. *Infect Immun* 1985;48:402-8.
- 8 Namavar F, Verwey-van Vught AMJJ, Bal M, MacLaren DM. Effects of *Bacteroides fragilis* cellular components on chemotactic activity of polymorphonuclear leukocytes towards *Escherichia coli*. *J Med Microbiol* 1987;24:119-24.
- 9 Nelson RD, Quie PG, Simmons RL. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J Immunol* 1975;115:1650-5.
- 10 Holdeman LV, Cato EP, Moore WEC. *Anaerobe laboratory manual*. 4th ed. Blacksburg, Virginia: Virginia Polytechnic Institute and State University, 1977.
- 11 Spiegel CA, Amsel R, Eschenbach D, Schoenknecht F, Holmes KK. Anaerobic bacteria in non-specific vaginitis. *N Engl J Med* 1980;303:601-7.
- 12 Fontaine EAR, Borriello SP, Taylor-Robinson D, Davies HA. Characteristics of a Gram-negative anaerobe isolated from men with non-gonococcal urethritis. *J Med Microbiol* 1984;17:129-40.
- 13 Spiegel CA, Roberts M. *Mobiluncus* gen nov, *Mobiluncus curtisii* subsp *curtisii* sp nov, *Mobiluncus curtisii* subsp *holmesii* subsp nov, and *Mobiluncus mulieris* sp nov, curved rods from the human vagina. *International Journal of Systematic Bacteriology* 1984;34:177-84.