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Sialidases (Neuraminidases) in Bacterial Vaginosis and Bacterial Vaginosis-Associated Microflora

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Bacterial vaginosis, *Prevotella* species, and *Bacteroides* species have been associated with prematurity and upper genital tract infection. *Prevotella* (*Bacteroides*) species and *Bacteroides fragilis* have also been associated with preterm birth. However, the mechanism by which lower genital tract infection causes upper genital tract disease remains poorly understood. Sialidases (neuraminidases) are enzymes which enhance the ability of microorganisms to invade and destroy tissue. Elevated levels of sialidase activity were detected in 42 (84%) of 50 vaginal fluid specimens from women with bacterial vaginosis and none of 19 vaginal fluids from women without bacterial vaginosis ($P < 0.001$). Vaginal fluid from women with bacterial vaginosis had a median specific activity of 9.8 U compared to 2.5 U of sialidase in women without bacterial vaginosis ($P < 0.001$). In order to determine the probable source of sialidases in vaginal fluid, the microorganisms recovered from women with bacterial vaginosis before and after treatment were assayed. Of 28 specimens from women with bacterial vaginosis, 27 (96%) yielded sialidase-positive bacteria, at a median concentration of $10^{6.5}$ CFU/ml of vaginal fluid. *Prevotella* and *Bacteroides* species accounted for the sialidase activity in 26 of the vaginal fluids, and *Gardnerella vaginalis* accounted for the sialidase activity in the remaining fluid. After treatment, sialidase was detected in the vaginal fluid of 1 (5%) of 22 women who responded to therapy and in all of 6 women for whom therapy failed. These data suggest that vaginal fluid sialidase is highly correlated with bacterial vaginosis and that the probable sources for this enzyme activity are the *Bacteroides* and *Prevotella* species present in the vagina.

Bacterial vaginosis (BV) is a syndrome defined microbiologically by decreased numbers of *Lactobacillus* spp. and increased numbers of *Gardnerella vaginalis*, *Bacteroides* spp., *Prevotella* spp., *Mobiluncus* spp., *Peptostreptococcus* species, and *Mycoplasma hominis* (7, 22). Accumulating evidence indicates that BV may be a risk factor for histologic chorioamnionitis (6), amniotic fluid infection (26, 29), postcesarean endometritis (29), and prematurity (14). Furthermore, three studies have linked vaginal *Prevotella* and *Bacteroides* spp. with preterm delivery (9, 15, 18). Although published data suggest that both BV and vaginal gram-negative anaerobic rods are risk factors for pregnancy complications (9, 14–16, 18, 26, 29), the pathogenesis remains poorly understood.

Several bacterial enzymes, including phospholipases and proteases, have been suggested as potential virulence determinants in prematurity (16, 17). Sialidases, formerly known as neuraminidases, are enzymes which cleave alpha-ketosidic linkages between the glycosyl residues of glycoproteins, glycolipids, or colominic acids and sialic acids. Sialidases have been implicated as virulence factors in several pathogenic organisms, including *Corynebacterium diphtheriae* and *Vibrio cholerae* (24), *Streptococcus pneumoniae* (8), and group B streptococci (2). Published data suggest that some of the organisms associated with bacterial vaginosis may also produce sialidases. Sialidase activity has been detected in anaerobic rods formerly included in the genus *Bacteroides* (25a), including *Prevotella bivia*, *Prevotella disiens*, black-pigmented *Prevotella* and *Porphyromonas* species, and *Bacteroides fragilis* group isolates (20). Sialidase activity was detected in 1 of 10 clinical isolates of *G. vaginalis* (28). Previous studies have demonstrated that several *Mycoplasma* spp. are able to use sialic acid receptors for adsorption (12, 13). However, in these investigations, isolates of *M. hominis* were not assayed for sialidase activity. Finally, no study to date has examined whether *Mobiluncus* or *Peptostreptococcus* species have sialidases. The association of sialidase activity with BV had not been previously examined.

The aims of this investigation were to assay vaginal fluid for sialidase activity and to compare the levels of sialidase activity in the vaginal fluid of women with BV with those detected in the vaginal fluid collected from women characterized as not having BV. In order to identify the probable source of the enzyme activity in vaginal fluid, vaginal isolates from women with BV, before and after treatment, were tested for sialidase activity.

MATERIALS AND METHODS

Women seeking care at the Seattle-King County Sexually Transmitted Disease Clinic at Harborview Medical Center in Seattle, Wash., were invited to participate in this study. All women provided written informed consent as approved by the University of Washington Human Subjects Review Committee. Fifty women with BV (defined by the presence of vaginal pH of >4.5, homogeneous vaginal discharge, odor in the presence of 10% KOH, and the presence of clue cells) were included for study. Nineteen women from the same clinic without vaginal symptoms and who were free of BV as diagnosed by clinical criteria were included as controls. All women were 18 to 24 years of age, not pregnant, and not hysterectomized, and none were menstruating at the time of

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the exam. Half of the women used oral contraceptives, and the remainder used barrier methods. The BV patients and controls did not differ with respect to these characteristics. Both BV patients and controls were culture negative for *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* and lacked signs and symptoms of yeast vaginitis.

Vaginal wash specimens were prepared by inserting a sterile, nonlubricated speculum, washing the vaginal walls with 3.5 ml of prereduced saline, and removing the resultant suspension with a sterile syringe. The vaginal wash specimens from 28 of the women with BV were diluted serially 1:10 and inoculated onto prereduced brucella agar supplemented with hemin, vitamin K, and 5% sheep blood for recovery of anaerobic gram-negative rods and onto human blood bilayer Tween agar for recovery of *G. vaginalis* as previously described (9). The remaining 22 vaginal wash specimens from women with BV and 19 specimens from women without infection were used for the quantitative assay of sialidase activity. To presumptively identify the source of sialidase activity among the microorganisms associated with BV, stocked culture isolates of *P. bivia*, *P. disiens*, *Mobiluncus* spp., *M. hominis*, *Peptostreptococcus* spp., and *G. vaginalis* collected during previous studies were assayed.

Sialidase activity was qualitatively determined by a filter paper spot test which has been described previously (19). A stock solution of 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Sigma, St. Louis, Mo.) was prepared in distilled water at a concentration of 110 μmol/ml and stored at −20°C. Prior to use, 20 μl of a 1.0 M sodium acetate buffer (pH 4.6) was added to 180 μl of the stock solution and mixed. Filter paper strips (Whatman no. 2; Whatman, Inc., Clifton, N.J.) saturated with substrate solution were inoculated with 5 μl of vaginal wash suspension and incubated for 15 min at 37°C. As a negative control, an aliquot of each vaginal wash was heated to 100°C for 30 min. None of the heat-treated samples had sialidase activity. *G. vaginalis* was propagated on Columbia agar supplemented with human blood. Anaerobic isolates were propagated on brucella agar supplemented with 5% sheep blood. The tests were interpreted by examining the strips under a long-wavelength (365 nm), hand-held mineral lamp. A fluorescent blue spot was indicative of sialidase activity.

The quantitative determination of sialidase activity with 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid was detected as described by Potier et al. (23) with a fluorescent Fluro IV model 1452 XII spectrophotometer (Gilford, Oberlin, Ohio) using excitation light at 365 nm and measuring emission light at 450 nm. A 10 mM stock solution of 4-methylumbelliferone (Sigma) was diluted to a 0.5 μM standard solution. Serial dilutions were made with 0.1 M sodium acetate buffer (pH 4.6) as a diluent and were assayed to create a standard curve.

To quantitatively assay the vaginal wash samples for sialidase activity, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid, at a concentration of 110 μmol/ml, was mixed 1:1 with 0.1 M sodium acetate buffer. Test sample (200 μl) was added to the reaction mixture and incubated at 37°C. After 15 min of incubation, 200 μl of a 1.33 M glycine buffer (pH 10.7) was added to the mixture to stop the reaction. A duplicate sample lacking substrate served as a control. Protein concentrations were determined using a method described by Lowry et al. (11) with bovine serum albumin (Sigma) as a standard. Specific activity was defined as micromoles of 4-methylumbelliferone formed per milligram of protein per minute at 37°C. All assays were performed in duplicate.

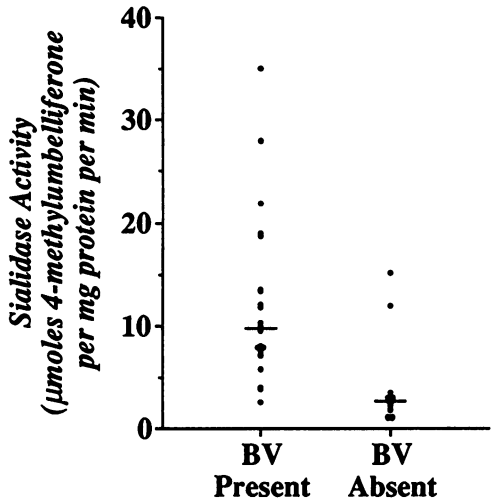

FIG. 1. Specific activity of sialidase in 22 women with and 19 women without BV. Median sialidase activity (horizontal bars) was 9.8 U in women with BV and 2.5 U in women without BV ($P < 0.001$ by the nonparametric median test).

RESULTS

When assayed by the spot test, 42 (84%) of 50 specimens from women with BV were positive for sialidase activity, compared to none of 19 specimens from women with normal vaginal flora ($P < 0.001$ by Fisher's exact test). Because of limitations in the quantity of vaginal wash specimens available from each woman, 41 vaginal fluid specimens (22 from women with BV and 19 from women with normal vaginal flora) were used for the quantitative measurement of sialidase activity. The remaining 28 washes from women with BV were assayed for sialidase by the spot test and inoculated onto plates for quantitative culture.

The specific activity of sialidase detected in vaginal washes from women with BV versus controls is shown in Fig. 1. The median sialidase activity among women with BV was 9.8 U (mean, 12.0 ± 8.1 U) versus 2.5 U (mean, 3.5 ± 3.6 U) in women without BV ($P < 0.001$ by the nonparametric median test). Specimens having less than 7 U of sialidase activity were negative for sialidase by the spot test.

In order to identify the probable source of sialidase activity among women with BV, pure stock-culture isolates of *P. bivia*, *P. disiens*, *G. vaginalis*, *Mobiluncus curtisii*, *Mobiluncus mulieris*, *Peptostreptococcus* species, and *M. hominis* were tested for sialidase activity. All of the 83 *P. bivia* isolates were positive for sialidase activity, compared with 12 (38%) of 32 *P. disiens* isolates. Twenty-one (20%) of 105 *G. vaginalis* isolates assayed were positive for sialidase activity. Isolates of *G. vaginalis* from women without BV were as likely to be positive for sialidases as isolates from women with BV (data not shown). None of the 10 *M. hominis* or 10 *Mobiluncus* species isolates tested were positive for sialidase activity. Four isolates each of *Peptostreptococcus asaccharolyticus*, *Peptostreptococcus anaerobius*, *Peptostreptococcus magnus*, *Peptostreptococcus prevotii*, and *Peptostreptococcus tetradius* were also negative for sialidase activity. These data suggested that anaerobic gram-

TABLE 1. Correlation between sialidase-positive vaginal bacteria and sialidase activity in vaginal fluid

BV diagnosis	No. (%) of women			Geometric mean concn (CFU/ml) of sialidase-positive bacteria in vaginal fluid
	Total	With sialidase detected in vaginal fluid	With sialidase-positive bacteria recovered from vaginal fluid	
Pretreatment	28	26 (93)	27 (96)	$10^{6.6}$
Posttreatment				
Clinical cure	22	1 (5)	7 (32)	$10^{4.4}$
Clinical failure	6	6 (100)	6 (100)	$10^{7.2}$
Recurrent	6	6 (100)	4 (67)	$10^{7.3}$

negative rods were the most probable source of sialidase activity in the vaginal fluid.

Quantitative cultures for anaerobic gram-negative rods, gram-positive cocci, and *G. vaginalis* were performed on 28 vaginal specimens from women with BV prior to treatment. Anaerobic gram-negative rods were recovered from 27 of 28 women, and *G. vaginalis* was recovered from all 28 women. Peptostreptococci were recovered from 25 (89%) of the 28 women. All isolates of *Prevotella* spp., *Porphyromonas* spp., and *Bacteroides* spp., and *G. vaginalis* recovered from these women were screened for sialidase activity. Sialidase-positive bacteria were recovered from 27 (96%) of the 28 vaginal specimens from women with BV. Twenty-six of the vaginal fluid specimens were positive for sialidase. Among these the sialidase activity was attributable to *P. bivia* in 17 (65%) cases, *Prevotella oralis* in one (4%) case, *Prevotella loeschii* in one (4%) case (all formerly belonging to the genus *Bacteroides* [25a]), *B. fragilis* in two (8%) cases, *Prevotella* species in four (15%) cases, and *G. vaginalis* in one (4%) case. The geometric-median concentration of these microorganisms was $10^{6.6}$ CFU/ml of vaginal fluid (range, 10^3 to 10^9 CFU/ml). One of the 28 vaginal fluid specimens had 10^3 CFU of sialidase-positive *Bacteroides vulgatus* per ml, but the vaginal fluid was negative for sialidase by the spot test.

Repeat vaginal cultures were obtained from the 28 women 1 to 3 weeks following completion of therapy with metronidazole or ampicillin. In addition, the vaginal fluid was tested for sialidase activity. Twenty-two (79%) of the women were clinically cured at follow-up (no clue cells, pH of ≤ 4.5, and no amine odor), and six had persistent BV (Table 1). Sialidase activity was detected in 1 (5%) of 22 vaginal specimens from women who had responded to therapy. Seven of the specimens from women who were free of BV following treatment and who did not have detectable sialidase in the vaginal fluid had sialidase-positive *P. bivia* recovered from the vaginal cultures (Table 1). In six of seven instances, *P. bivia* was present at concentrations of $<10^5$ CFU/ml, and in the remaining specimen *P. bivia* was present at 10^7 CFU/ml. Thus, the presence of detectable sialidase in the vaginal fluid was usually correlated with $>10^5$ CFU of sialidase-positive, anaerobic, gram-negative rods per ml.

All of the six specimens from women who failed therapy were positive for sialidase; four specimens yielded *P. bivia* at a median concentration of $10^{6.8}$ CFU/ml, and the remaining two specimens yielded *B. fragilis* at $10^{8.0}$ CFU/ml and a *Prevotella* species at 10^9 CFU/ml.

At a third follow-up 1 month after therapy, six women developed recurrent BV. Sialidase was detected in all of the vaginal fluid specimens, and sialidase-positive *P. bivia* and *P. disiens* were present at a median concentration of $10^{7.3}$ CFU/ml.

DISCUSSION

The aims of this investigation were to determine whether sialidases in vaginal fluid were associated with BV and to identify which organisms recovered from women with BV contributed sialidase activity to the vaginal fluid. In this study, we demonstrated that 84% of women with BV have detectable sialidases in their vaginal fluid and that the specific activity is higher in these women than in women without the syndrome. Our data further implicate anaerobic gram-negative rods belonging to the genera *Prevotella* and *Bacteroides* as the major source of sialidase activity in the vaginal fluid.

Prevotella (formerly *Bacteroides*) species at concentrations of 10^7 to 10^8 CFU/ml of vaginal fluid can be recovered from the vaginae of 94% of women with BV (4). In the present study, clinical cure was associated with an 88% reduction of sialidase activity in the vaginal fluid and a 64% reduction in the frequency of sialidase-positive vaginal bacteria, as well as a 100-fold decrease in their concentration. Conversely, clinical failure was correlated with persistence of vaginal fluid sialidase and the persistence of sialidase-positive anaerobes in the vaginal fluid. These data suggest that the presence of sialidase activity in the vaginal fluids of women with BV is associated with the presence of sialidase-positive *Prevotella* and *Bacteroides* species.

Prevotella and *Bacteroides* species are able to ascend into the upper genital tract to cause infection during pregnancy. Women with BV are significantly more likely to develop intraamniotic infection (26) and postpartum endometritis (29). Vaginal colonization with *Prevotella* species has been linked to an increased risk of preterm delivery and preterm premature rupture of membranes in two studies (15, 18). In a recent study, *P. bivia*, isolated from the vagina at concentrations greater than 10^4 CFU/ml of vaginal fluid, was associated with a twofold increased risk of preterm delivery among women in preterm labor (9). *P. bivia* has been recovered from the amniotic fluid of 46% of women with intra-amniotic infection who delivered low-birthweight infants (27). *B. fragilis* in the vagina has also been linked to an increased risk of preterm birth (9). Anaerobic gram-negative rods have also been recovered from the chorioamnion and are associated with histologic chorioamnionitis (5).

The production of sialidase by the anaerobes associated with prematurity suggests that the enzyme may be involved in their pathogenesis. Studies have demonstrated that exposure of tissues to sialidases eliminates subterminal sugars, resulting in an increased adherence capability by certain bacteria (3, 25). Since both cervical mucus and amniotic fluid have been demonstrated to contain significant amounts of sialic acid (10, 21), sialidases may promote virulence by enhancing the ability of these organisms to adhere to, invade, and destroy mucosal tissue. Bacterial sialidases also decrease collagen synthesis in fibroblasts (1).

The other microorganisms associated with BV include *G. vaginalis*, *Mobiluncus* spp., *Peptostreptococcus* spp., and *M. hominis* (7, 14). In the present study, these genera did not usually produce detectable sialidase activity. *G. vaginalis* accounted for the vaginal sialidase activity in only 1 of 28 women with BV.

Further studies are needed to determine whether there is a relationship between sialidase activity in vaginal fluid and

prematurity, upper genital tract infection, and BV. It will be of interest to determine whether sialidases in the vaginal fluid of pregnant women are associated with an increased risk of preterm labor or invasion of the amniotic cavity.

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