A Novel Bacterial Mucinase, Glycosulfatase, Is Associated with Bacterial Vaginosis

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The modifications to the vaginal habitat accompanying a change to vaginal flora in bacterial vaginosis (BV) are poorly understood. In this study enzymes involved in mucin degradation were measured, including a novel glycosulfatase assay. Women attending an emergency walk-in sexually transmitted disease clinic were studied. One high vaginal swab (HVS) was used to prepare a gram-stained smear to determine BV status, using Ison and Hay’s criteria, and a separate swab was used for the purposes of the assays. The median glycosulfatase activity was 8.5 (range, 1.2 to 31.9) nmol h⁻¹ 1.5 ml⁻¹ of HVS suspension in patients with BV compared to 0.5 (range, 0.7 to 9.4) nmol h⁻¹ 1.5 ml⁻¹ of HVS suspension in patients without BV (P < 0.001). The median glycoprotein sialidase activity was 29.2 (range, 17 to 190) nmol h⁻¹ 1.5 ml⁻¹ of HVS suspension in patients with BV compared to 1.1 (range, 41 to 48) nmol h⁻¹ 1.5 ml⁻¹ of HVS suspension in patients without BV (P < 0.001). A rapid spot test for sialidase was positive in 22/24 patients with BV (sensitivity, 91.7%; 95% confidence interval [CI], 73 to 99%) and negative in 32/35 patients without BV (specificity, 91.4%; 95% CI, 76.9 to 98.2%) (P < 0.001). Glycosulfatase activity significantly correlated with both glycoprotein sialidase activity and the sialidase spot test (P = 0.006 and P < 0.001, respectively). The results are consistent with the hypothesis that the consortium of bacteria present in BV requires the ability to break down mucins in order to colonize the vagina and replace the normal lactobacilli.
**MATERIALS AND METHODS**

Collection of clinical samples. Sixty-one women attending an emergency walk-in clinic at the Milne Centre for Sexual Health who required a vaginal speculum examination were studied after informed consent was obtained. The age range of the women was 16 to 50 years. An HVS was obtained at speculum examination and placed into 1.5 ml Tris chloride buffer (25 mM, pH 7.4). Swabs were immediately vortexed in the buffer for 2 min, the swab squeezed out and removed, and the suspension placed on ice.

A separate swab was taken for microscopic diagnosis of BV. The swab was rolled onto a glass slide, air dried, heat fixed, and gram stained. Slides were classified according to Ison and Hay’s criteria (10) as predominantly lactobacillus flora (grade I), mixed lactobacillus and BV-related organisms (grade II), or few or no lactobacilli and predominantly BV organisms present (grade III) (6, 25, 31). Slides were read independently by P. Horner and T. Crowley and results compared. Discordant findings were reviewed to reach agreement. Cathy Ison (Health Protection Agency, London) reviewed 10 slides on which there was ongoing debate. These were sent together with 10 slides on which there was consensus agreement. There was complete agreement between Ison’s and our assessments of the latter 10 slides. Ison assessed all slides without knowledge of their identity, and her classification was taken as final.

For the purposes of the analyses, BV was defined as grade III flora and grades I and II were defined as not having BV.

**Quantitative glycosulfatase assay using 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside 6-sodium sulfate (SO3-6-GlcNAc-1-PNP) substrate.** The enzyme assay involves the desulfation of SO3-6-GlcNAc-1-PNP by the glycosulfatase activity to convert GlcNAc-1-PNP produced by the bacterial glycosulfatase activity to PNP. Then, 170 μl of sodium glycine buffer (0.5 M, pH 9.6) was added to all wells and the suspension placed on ice.

Table 1. Sialidase and sulfatase activities found in HVSs from subjects with BV and subjects without BV

<table>
<thead>
<tr>
<th>Enzymatic activity measured</th>
<th>Value for group (nmol h⁻¹ 1.5 ml⁻¹ of HVS suspension)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoprotein sialidase activity</td>
<td>Subjects with BV</td>
</tr>
<tr>
<td>Median range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Glycoprotein sialidase activity</td>
<td>29.2 (17–190)</td>
</tr>
<tr>
<td>Sulfatase activity</td>
<td>8.5 (1.2–31.9)</td>
</tr>
</tbody>
</table>

*Significance of data, analyzed by the Mann-Whitney U test.

Assays were carried out in 96-well plates. SO3-6-GlcNAc-1-PNP (15 μL, 3.2 mM) and HVS suspension (15 μL) were added to duplicate wells (i). Tris chloride buffer, pH 7.4, was present at a 67 mM final concentration. Three controls were incubated in parallel, containing (ii) HVS suspension and buffer with no substrate (in duplicate), (iii) substrate and buffer without HVS suspension (in quadruplicate), and (iv) buffer alone (in quadruplicate). The contents of wells were mixed, placed in a moist chamber, and incubated at 37°C overnight with gentle circular rotation (50 rpm). After 18 to 22 h, 10 μl of Aspergillus oryzae extract (Sigma G7138) with a hexosaminidase activity sufficient to form 1.5 nmol product min⁻¹ was added to wells i and iii and incubated for a further hour to convert GlcNAc-1-PNP produced by the bacterial glycosylase activity to GlcNAc and PNP. This choice of hexosaminidase source is crucial, since some commercial hexosaminidases will slowly remove PNP from SO3-6-GlcNAc-1-PNP. Then, 170 μl of sodium glycine buffer (0.5 M, pH 9.6) was added to all wells, and the absorbance difference was read between 405 nm (maximum absorbance of the PNP anion) and 620 nm (reference wavelength) using a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA). The absorbance due to the PNP production was corrected for light scattering and possible substrate absorbance by the auxiliary enzyme by subtracting the combined absorbances of well ii plus well iii from well i plus well iv and averaging the multiple readings.

**Sialidase spot test assay.** This was a modification of the previously described sialidase filter paper spot test (31) using 5-bromo-4-chloro-3-indolyl-acetate/neuraminidase substrate. A 3-mm disk of Whatman no. 1 filter paper was placed in the wells of a 96-well plate. The substrate (15 μl of 1.57 mM BCIN in 25 mM Tris chloride buffer, pH 7.4) and 10 μl of HVS suspension were added, and the plate was incubated at 37°C overnight (about 21 h) in a water-saturated atmosphere. The blue color on the filter due to 5-bromo-4-chloro-3-indole formation was estimated semiquantitatively, from 0 to +++. The color could be judged after 1 h, but it was convenient to record all of the spot test assays at one time, 3 h after the first subject’s test was initiated.

**Sulfatase activity in HVSs.** The 24 subjects with BV had a median glycosulfatase activity of 8.5 (range, −1.2 to 31.9) nmol PNP h⁻¹ 1.5 ml⁻¹ of HVS suspension, while the 35 subjects without BV had a median activity of 0.5 (range, −0.7 to 9.4).

**RESULTS**

Of the 61 women in the study, 31 were graded as not having BV (grade I), 4 had intermediate flora (grade II), and 24 were graded as having BV (grade III). Two subjects were subsequently excluded from the trial, one because of an enzyme assay error and one because the HVS sample did not contain sufficient cells for diagnosis.

**Statistical methods.** Data collected on the enzyme activities of HVSs from subjects with BV and subjects without BV were analyzed using SPSS version 12. Two samples were excluded from the study, since their data were incomplete.

Both glycosulfatase activity and glycoprotein sialidase activities in HVSs appeared to have a log normal distribution; however, due to the presence of zero and negative values, the data were not transformed. Medians and ranges have been used to describe the data; however, means and standard deviations have also been included to facilitate any future sample size calculations. Correlations between continuous variables have been carried out using Spearman’s rank correlation coefficient, or Kendall’s tau in the case of ordered categorical variables, and scatter plots and box plots have been used to demonstrate the clinical significance.

To determine the statistical significance of differences between samples from subjects with BV and subjects without BV, a Mann-Whitney test was used, with data from intermediate (grade II) patients being included with data from subjects without BV.

Confidence intervals were calculated for sensitivities and specificities with STATA version 7, using the exact binomial method.

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nmol PNP h⁻¹ 1.5 ml⁻¹ of HVS suspension (Table 1; Fig. 1). The sulfatase activities of the HVSs from the two groups were significantly different as analyzed by the Mann-Whitney U test (P < 0.001).

Glycoprotein sialidase activities in HVSs. The 24 subjects with BV had a median activity of 29.2 (range, 17 to 190) nmol sialic acid h⁻¹ 1.5 ml⁻¹ of HVS suspension, while the 35 subjects without BV had a median activity of 1.1 (range, 41 to 48) nmol sialic acid h⁻¹ 1.5 ml⁻¹ of HVS suspension (Table 1; Fig. 1). The AGP sialidase activities of the HVSs from the two groups were significantly different as analyzed by the Mann-Whitney U test (P < 0.001).

Glycoprotein sialidase activity compared to glycosulfatase activity. Comparison of the sialidase and glycosulphatase activities (Fig. 1) showed that only the individuals with BV showed a positive correlation. Cases without BV yielded a Spearman’s rank correlation coefficient of −0.22 (P = 0.20), while a value of 0.54 (P = 0.006) was found for the group with BV.

Sialidase spot test for BV in HVSs. In the present study, this test was slightly modified for use in a 96-well microtiter plate. The same fresh HVS samples were qualitatively assayed for BCIN sialidase activity in parallel with the quantitative glycoprotein sialidase test. A positive spot test was observed for 22 out of 24 subjects with BV (sensitivity, 91.7%; 95% confidence interval, 73 to 99%) and a negative spot test for 32 of 35 subjects without BV (specificity, 91.4%; 95% confidence interval, 76.9% to 98.2%) (P < 0.001, chi square).

Correlation between sialidase spot test and sulfatase activity. Results of these two tests were significantly correlated, with a Kendall’s tau coefficient of 0.573 (P < 0.001) (Fig. 2).

Correlation between sialidase spot test and sialidase activity. Results of these two tests were significantly correlated, with a Kendall’s tau coefficient 0.567 (P < 0.001) (Fig. 3).

DISCUSSION

The novel finding in the present work is that a further enzyme involved in the overall process of mucin degradation is highly associated with BV. Glycosulfatase activity was significantly associated with BV. This enzyme has not previously been described in the microflora from the lower genital tract of women and can now be detected using a novel and specific substrate. In addition, the glycosulfatase activity from cases with BV was correlated with sialidase activity. The data presented suggest that both sialidase and glycosulfatase activities are important in the pathogenesis of BV. Both of these enzymes belong to families with multiple members showing a variety of specificities. This emphasizes the need to select and test substrates which will reflect the likely targets in the mucosal protective barrier.

This is a small study of patients at high risk of having a sexually transmitted infection (STI) who were attending an
emergency clinic, BV is associated with STIs, including both C. trachomatis and N. gonorrhoeae, and it is therefore possible that other STIs could be confounding. The numbers with either of these infections was too small (six) to control for in the analyses. However, it is well documented that sialidase (1, 31, 33), hexosaminidase, galactosidase (8), and proline iminopeptidase (16, 17, 22) activities in the vaginal flora increase markedly in the majority of subjects diagnosed as having BV. Furthermore, the strong correlation of glycosulfatase activity with sialidase activity in the cases with BV and not in the individuals without BV suggests that the increased mucinase activities observed were indeed a consequence of BV and not due to other confounding STIs. The sulfatase assay clearly has potential to serve as a diagnostic marker for bacterial vaginosis. This needs to be assessed in a larger study involving the general population.

Further analysis of the glycosulfatase activity is now needed. The mechanism of PNP release from the assay substrate, SO₃⁻-population, needs to be assessed in a larger study involving the general population. To serve as a diagnostic marker for bacterial vaginosis. This observed were indeed a consequence of BV and not due to other confounding STIs. The sulfatase assay clearly has potential to serve as a diagnostic marker for bacterial vaginosis. This needs to be assessed in a larger study involving the general population.

Prevention of vaginal colonization by bacteria associated with BV is believed to be due to the mucosal surface environment, including low pH, antimicrobial molecules secreted by the host and lactobacilli (5, 29), and the mucus and mucosal barrier. The present results implicate participation of the cervical mucins, glycoproteins, and glycolipids at the mucosal surface in BV pathology. Bacterial mucolytic activity in the vaginal habitat of women with BV indicates that these molecules are being actively altered or degraded. However, there is no evidence explaining how changes to these glycoconjugates contribute to the disease.

Several recent studies have demonstrated interactions of bacteria at mucosal surfaces and are pertinent to colonization by BV-associated bacteria. The role of mucins, glycoproteins, and glycolipids in the mucosal barrier as receptors in Helicobacter pylori adherence and persistence mechanisms in the stomach has been established. These molecules carry Lewis antigens as glycan ligands recognized by specific H. pylori adhesins (9, 13). Furthermore, the presence of mucins rich in terminal α-linked N-acetylglucosamine located in the gastric glands has been linked with a “natural antibiotic action” and explains why H. pylori is unable to colonize deep in the gastric glands (11). In the murine intestine selectively colonized with Bacteroides thetaiotaomicron, an adaptation to the dietary situation has been shown at the genomic level. Upregulation of the mucolytic enzymes is observed when polysaccharides are removed from the habitat and the endogenous host mucus becomes an alternative energy source (24). These examples highlight the significance of the glycoconjugates in the mucosal protective barrier and the need for more detailed structural information. In particular, a closer analysis of the glycan complement presented at the cervical and vaginal mucosal surfaces during the menstrual cycle (23) will be necessary to evaluate the physiological action of the mucinase activities released in BV. The results suggest that glycosulfatase can be added to the group of mucinase enzymes, including sialidase, hexosaminidase, galactosidase, and proline iminopeptidase, associated with BV. Each of these enzymes has a specificity that correlates with different stages in the process of mucin degradation (33). The results of this study are consistent with the hypothesis that the consortium of bacteria present in BV requires the ability to break down mucins in order to colonize the vagina and replace the normal lactobacilli. In addition, changes in the physicochemical character of mucins as a result of this combined enzymatic degradation, including loss of negative charge, may lead to decreased effectiveness of the mucus barrier and its role in excluding pathogenic microorganisms, such as C. trachomatis and N. gonorrhoeae.

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Informed consent was obtained from all patients used in this study. We have no commercial or other association that might pose a conflict of interest.

REFERENCES


