Local and Systemic Cytokine Levels in Relation to Changes in Vaginal Flora

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Background. Bacterial vaginosis (BV) is associated with increased risk of obstetrical and gynecologic complications and acquisition of sexually transmitted diseases. Despite this, very little is known about the pathogenesis of this disease.

Methods. Interleukin (IL)-1β, tumor necrosis factor-α, IL-6, and IL-8 concentrations in vaginal wash and serum samples from women with normal flora, intermediate flora, and BV (determined by Nugent criteria) were measured by enzyme-linked immunosorbent assay.

Results. Cytokine levels were not different between women with intermediate flora and women with BV. Women with either intermediate flora or BV had significantly higher concentrations of IL-1β in vaginal wash samples than did women with normal flora. The presence of IL-1β in vaginal wash samples was associated with >30 Gardnerella or Prevotella morphotypes per high-power field, as detected by Gram staining of vaginal swab specimens. Variation in the numbers of Lactobacillus and Mobiluncus species did not influence local cytokine levels. Serum cytokine levels were not influenced by any changes in vaginal flora.

Conclusions. Women with intermediate flora generate significant cytokine responses. It is possible that the risks associated with BV may also affect women with intermediate flora and that appropriate treatment may reduce such risk.

Bacterial vaginosis (BV) is the most common cause of vaginal symptoms among women of childbearing age. BV has serious consequences, including increasing the likelihood of acquiring sexually transmitted infections, such as HIV infection, and obstetrical and gynecologic complications, including preterm delivery [1-8]. The pathogenic mechanisms of BV remain relatively unexplored. Unlike vaginitis caused by either Candida species or Trichomonas vaginalis, BV is not attributable to infection with a single pathogenic organism. Instead, BV has a complex etiology that includes log10-fold increases in the number of facultative anaerobic bacteria (including combinations of Gardnerella vaginalis, Bacteroides species, Peptostreptococcus species, and Prevotella species) and a concomitant substantive decline and eventual disappearance of H2O2-producing Lactobacillus species from the vaginal flora [9-11]. In addition, Mobiluncus species are also highly associated with BV and are rarely found in women with normal (healthy) vaginal flora [12-14].

From a clinical viewpoint, BV is not normally associated with overt inflammatory responses. BV does, however, cause endometritis and cervical inflammation, including infiltration of neutrophils and localized erythema. In addition, women with BV have higher concentrations of interleukin (IL)-1β and IL-8 in vaginal fluid than do women with normal flora [15-18]. Pregnant women with BV also produce a similar profile of vaginal cytokines [19, 20]. Other studies, however, have failed to show associations between specific cytokines, such as IL-6, and BV [21, 22]. Direct cause and effect has not been demonstrated; however, the production of inflammatory cytokines by women with BV and the known effects of these proteins on parturition and immune and inflammatory processes is consistent with the serious complications associated with BV.

Significant changes in vaginal flora occur before a diagnosis of BV. In women with intermediate flora,
the numbers of anaerobic gram-negative bacteria, such as Gardnerella and Prevotella species, are elevated, whereas the numbers of protective Lactobacillus species are decreased, compared with those in women with normal flora. We postulated that cytokine responses might occur in these women before the diagnosis of BV. The aims of this study were to measure the levels of mucosal and systemic cytokines in women with intermediate vaginal flora and compare them with those in women with normal flora and women with BV, to identify whether cytokine production could be attributable to specific changes in the bacterial types used to diagnose BV by Gram staining.

**PATIENTS, MATERIALS, AND METHODS**

**Patients and samples.** Women attending the Jefferson County Sexually Transmitted Diseases Clinic were recruited into a prospective longitudinal study of BV and sexually transmitted diseases. At enrollment and at all subsequent visits, all subjects were screened for T. vaginalis (InPouch; BioMed), Neisseria gonorrhoeae, and Chlamydia trachomatis (PACE 2; Gen-Probe). Patients with any of these sexually transmitted infections were promptly treated according to established guidelines [23]. Patients were evaluated for their BV status by microscopic evaluation of the relative numbers of Lactobacillus morphotypes, Gardnerella or Prevotella morphotypes, and gram-negative curved rods (Mobiluncus species) present in vaginal smears (Nugent criteria [24]), and samples were collected. Vaginal wash (5 mL of sterile saline) and serum specimens were collected after the diagnostic samples and were then stored at 4°C until being transported to the laboratory (on the same day). At the laboratory, serum and vaginal wash specimens were aliquoted and stored at −80°C until being assayed. Some serum specimens were used up in the course of previous studies (7 of 44 from subjects with normal flora, 1 of 32 from subjects with intermediate flora, and 6 of 91 from subjects with BV) and were not available for assay; therefore, they were not included in the present results. Informed consent was obtained from all patients, with approval of the Committee on the Protection of the Rights of Human Subjects at the University of Alabama at Birmingham.

**Quantitation of cytokine concentrations.** Cytokine concentrations in serum and vaginal wash specimens were quantified by ELISA, in accordance with the manufacturer’s instructions. Briefly, 96-well ELISA plates were coated overnight with anti–human IL-1β, anti–human TNF-α, anti–human IL-6, or anti–human IL-8 (all from R&D Systems) in PBS. After unbound coating antibody was removed and nonspecific protein binding was blocked, duplicate serial dilutions of sample or standard (recombinant human IL-1β, TNF-α, IL-6, or IL-8; 2 ng/mL−8 pg/mL) were incubated overnight at room temperature. After overnight incubation, captured cytokines were detected with mouse anti–human IL-1β, TNF-α, IL-6, or IL-8, followed by incubation with peroxidase-conjugated anti–mouse IgG (Southern Biotechnology Associates) for 4 h. For all assays, color development used a substrate consisting of o-phenylenediamine and H₂O₂ in citrate-phosphate buffer (pH 4.0); development was stopped with 1 mol/L sulfuric acid after 15 min, and absorbance was read at 490 nm in a Vmax microplate reader (Molecular Devices) interfaced to a Macintosh computer for data retrieval. Standard curves were determined for each plate and type of assay from serial dilutions of human immunoglobulin calibrator at appropriate concentrations. Unknowns were interpolated on standard curves generated by a computer program with 4-parameter logistic algorithms, in which parallelism between unknown and standard dilution curves was demonstrated over the range of unknown dilutions used for calculation.

**Statistics.** Cytokine levels, subjects’ ages, vaginal pH, and Nugent scores were compared across multiple groups (women with normal flora, intermediate flora, or BV) by use of the Kruskal-Wallis and Dunn’s post tests. The Mann-Whitney U test was used for comparisons between 2 groups. Comparisons of the number of subjects with positive results for C. trachomatis, N. gonorrhoeae, or T. vaginalis infection or the presence of vaginal discharge or clue cells were made with the χ² test for multiple groups. For analyses of the effects of changes in Nugent component score, the results from 4 groups with 2 similar bacterial profiles and small sample sizes were combined, to facilitate statistical analyses. Data from women with no changes in the numbers of either Lactobacillus or Mobiluncus morphotypes (scores of 0) and with Gardnerella or Prevotella scores of 2 or 3 (i.e., some increase) were combined. Similarly, data from women with Gardnerella or Prevotella scores of 4 (the maximum score for those bacterial morphotypes), no changes in the numbers of Mobiluncus morphotypes (score of 0), and Lactobacillus scores of 1 or 2 (i.e., some decrease) were combined. All statistical tests were performed using the Prism software package (version 3.03; Graphpad Software).

**RESULTS**

**Description of study population.** Vaginal wash and serum specimens were obtained from 44 women with normal flora, 32 women with intermediate flora, and 91 women with BV (table 1). The patient population was 97% African American and 3% white, which corresponds to the population of clinic attendees at the Jefferson County Health Department. None of these patients were pregnant. The median age at enrollment for all subjects was 23 years (range, 18–44 years). There were no significant differences in the subjects’ ages; in the number of subjects infected with C. trachomatis, N. gonorrhoeae, or T. vaginalis; or in the number of subjects with vaginal discharge between the women with normal flora, intermediate flora, and BV. The number of subjects with a vaginal yeast infec-
tion trended higher in women with intermediate flora, but the difference did not reach significance ($P = .0648$, $\chi^2$ test for multiple groups). As expected, Nugent score, vaginal pH, and the presence of clue cells were significantly different in each diagnostic subject group.

**Vaginal cytokine concentrations in relation to diagnosis.** IL-1β concentrations were >0 pg/mL in 25 (57%) of 44, 30 (94%) of 32, and 88 (97%) of 91 vaginal wash samples from women with normal flora, intermediate flora, and BV, respectively (figure 1). IL-1β concentrations were significantly higher in women with either intermediate flora (median, 89 pg/mL; $P < .01$) or BV (median, 91 pg/mL; $P < .001$) than in women with normal flora (median, 17 pg/mL). IL-8 concentrations were >0 pg/mL in 33 (75%), 25 (78%), and 75 (82%) vaginal wash samples from women with normal flora, intermediate flora, and BV, respectively. Median IL-8 concentrations were 65, 126, and 169 pg/mL, respectively. IL-8 concentrations were significantly higher in women with BV than in women with normal flora ($P < .05$). TNF-α concentrations were >0 pg/mL in 20 (45%), 13 (41%), and 40 (44%) vaginal wash samples from women with normal flora, intermediate flora, and BV, respectively. Median TNF-α concentrations were 0 pg/mL for all groups. IL-6 concentrations were >0 pg/mL in 27 (61%), 20 (63%), and 65 (71%) vaginal wash samples from women with normal flora, intermediate flora, and BV, respectively. Median IL-6 concentrations were 73, 186, and 170 pg/mL, respectively.

**Serum cytokine concentrations in relation to diagnosis.** IL-1β concentrations were >0 pg/mL in 21 (57%) of 37, 17 (55%) of 31, and 62 (73%) of 85 serum samples from women with normal flora, intermediate flora, and BV, respectively. Median IL-1β concentrations were 12, 24, and 20 pg/mL, respectively. TNF-α concentrations were >0 pg/mL in 15 (41%), 16 (52%), and 43 (51%) serum samples from women with normal flora, intermediate flora, and BV, respectively. Median TNF-α concentrations were 0, 17, and 19 pg/mL, respectively. IL-6 concentrations were >0 pg/mL in 25 (68%), 20 (65%), and 57 (67%) serum samples from women with normal flora, intermediate flora, and BV, respectively. Median IL-6 concentrations were 188, 176, and 165 pg/mL, respectively. IL-8 concentrations were >0 pg/mL in 4 (11%), 9 (29%), and 24 (28%) serum samples from women with normal flora, intermediate flora, and BV, respectively. Median IL-8 concentrations were 0 pg/mL in all groups.

**Relationship between individual Nugent-defined bacterial component scores and cytokine concentrations.** Cytokine concentrations in vaginal wash and serum samples were compared after stratification of the sample population on the basis of changes in the individual component scores that constitute the Nugent score. Table 2 shows median and 25th-75th percentile cytokine levels in vaginal wash and serum samples. There was significant variance in the levels of IL-1β in vaginal wash specimens after stratification for component score ($P = .0013$, Kruskal-Wallis test). IL-6 and IL-8 showed a similar trend; however, the differences did not quite reach statistical significance ($P = .0587$ and $P = .0754$, respectively). TNF-α levels did not vary by component score.

Our observations of the medians and distribution of IL-1β levels in vaginal wash specimens suggested a possible relationship between higher levels of IL-1β in vaginal wash samples and the presence of high numbers of Gardnerella or Prevotella morphotypes (component score of 4; >30/high-power field) as determined by Gram staining of vaginal smears. On the basis of that hypothesis, IL-1β levels from subjects with either no

Table 1. Characteristics of and signs of infection in subjects, stratified by composition of vaginal flora.

<table>
<thead>
<tr>
<th>Characteristic or sign of infection</th>
<th>Normal ($n = 44$)</th>
<th>Intermediate ($n = 32$)</th>
<th>Bacterial vaginosis ($n = 91$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), years</td>
<td>23 (18-44)</td>
<td>24 (18-37)</td>
<td>23 (18-41)</td>
</tr>
<tr>
<td>Chlamydia trachomatis</td>
<td>3 (7)</td>
<td>4 (13)</td>
<td>7 (6)</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>1 (2)</td>
<td>4 (13)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>Trichomonas vaginalis</td>
<td>3 (7)</td>
<td>3 (9)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>Discharge</td>
<td>26 (59)</td>
<td>23 (72)</td>
<td>69 (76)</td>
</tr>
<tr>
<td>Yeast</td>
<td>7 (16)</td>
<td>10 (31)</td>
<td>12 (13)</td>
</tr>
<tr>
<td>Nugent score, a median (range)</td>
<td>1 (0-3)</td>
<td>5 (4-6)</td>
<td>8 (7-10)</td>
</tr>
<tr>
<td>pH, b median (range)</td>
<td>4.0 (4.0-6.5)</td>
<td>4.7 (4.0-7.0)</td>
<td>5.5 (4.4-7.0)</td>
</tr>
<tr>
<td>Clue cells b</td>
<td>2 (5)</td>
<td>14 (44)</td>
<td>81 (69)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of subjects positive for the sign of infection, unless otherwise noted.

a $P < .0001$, Kruskal-Wallis test. All groups were significantly different from each other ($P < .05$, Dunn's post-test).

b $P < .0001$, $\chi^2$ test for multiple groups.
Gardnerella or Prevotella morphotypes (component score of 0) or some Gardnerella or Prevotella morphotypes (component score of 2 or 3) were compared with IL-1β levels from each stratified group in which high numbers of Gardnerella or Prevotella morphotypes (component score of 4) were present (figure 2). IL-6, IL-8, and TNF-α levels were not analyzed in this fashion, because the variances between groups were not significantly different in previous analyses. IL-1β levels in vaginal wash specimens were higher \((P < .05, \text{Dunn's post tests following the Kruskal-Wallis test})\) in 3 stratified groups with high numbers of Gardnerella or Prevotella morphotypes \((0-4-0, 3-4-0, \text{and } 4-4-2)\), compared with those in either group in which the numbers were lower \((0-0-0 \text{ or } 0-2,3-0)\). Vaginal IL-1β levels in the remaining 2 groups \((1,2-4-0 \text{ and } 4-4-0)\) showed a similar trend, compared with those in groups with no or low numbers of Gardnerella or Prevotella morphotypes, but the differences did not quite reach statistical significance.

In addition to the analyses of the stratified groups, IL-1β levels in vaginal wash samples were compiled for samples in which the Gardnerella or Prevotella score was 4 and then compared with the compiled IL-1β levels in samples in which the score was <4. Levels of IL-1β in vaginal wash specimens were higher in groups in which the Gardnerella or Prevotella component score was 4 than in groups in which fewer or no Gardnerella or Prevotella morphotypes were identified by vaginal smear \((P < .0001, \text{Mann-Whitney } U \text{ test})\). In contrast, IL-1β
levels in vaginal wash specimens were not different when samples with high or low component scores for either *Lactobacillus* or *Mobiluncus* species were compared. Serum cytokine levels were not affected by changes in any of the Nugent component scores.

**DISCUSSION**

For the most part, studies addressing the pathogenic mechanisms of BV have focused on the microbiological changes in vaginal flora that characterize this complex disease. Although the host response to BV has not been overlooked, few published reports have addressed this aspect of the pathogenesis of BV. In this study, we have measured the concentrations of cytokines associated with inflammation and parturition in vaginal wash and serum specimens from women with normal flora, intermediate flora, and BV. Our results suggest novel directions with respect to the pathogenesis of BV and the treatment of abnormal vaginal flora.

Median levels of IL-1β, TNF-α, IL-6, and IL-8 in vaginal wash specimens were of similar magnitude in women with intermediate flora and women with BV, and we did not detect any statistical differences in the levels of any cytokine between these 2 groups. It must be noted, however, that these results need to be replicated in larger populations to demonstrate that the groups are not statistically different. In contrast, women with either intermediate flora or BV had significantly higher concentrations of IL-1β in vaginal wash specimens than did women with normal flora. Vaginal IL-8 levels were also higher in women with BV than in women with normal flora. Although not statistically significant in this population, the data trend suggests that IL-6 levels may also be significantly higher in vaginal wash specimens from women with either intermediate flora or BV than in specimens from women with normal flora. Similarly, IL-8 levels may also be significantly higher in women with intermediate flora than in women with normal flora. TNF-α was detected in some vaginal wash samples. TNF-α levels, however, could not be attributed to changes in vaginal flora.
likely role played by inflammatory cytokines in the serious complications associated with BV, the production of the same level and profile of cytokines by women with intermediate flora suggests that treatment of intermediate flora may reduce the incidence of the adverse health outcomes that are currently associated only with BV. Second, local cytokine production appears to be triggered when the numbers of vaginal Gardnerella or Prevotella morphotypes reach levels that are consistent with a Nugent component score of 4 (>30 bacteria/high-power field) on a Gram-stained vaginal smear. This suggests that the presence of Gardnerella or Prevotella morphotypes at >30 bacteria/high-power field in a vaginal smear may be of sufficient diagnostic value to initiate treatment of the patient before the loss of Lactobacillus species or the appearance of Mobiluncus species—changes that are normally required for the laboratory diagnosis of BV (i.e., Nugent score of ≥7).

Our current understanding of BV suggests that the disease results from a continuum of changing bacterial flora (species and relative numbers) rather than from the activities of a single pathogen. Because of the flexible nature of the disease process in BV, the host response has to be considered and analyzed throughout a spectrum of changes in vaginal bacteria. The Nugent criteria, with semiquantitative measurements of several important vaginal species associated with BV (i.e., Gardnerella or Prevotella species, Lactobacillus species, and Mobiluncus species) provide such a spectrum. Therefore, we stratified vaginal and systemic cytokine levels in relation to changes in the component scores that are combined to create the Nugent score. We found that local cytokine levels were related to the presence of high, but not moderate, numbers of vaginal anaerobic gram-negative bacteria (Gardnerella or Prevotella morphotypes). In contrast, cytokine levels were not affected by the changes in the numbers of either Lactobacillus or Mobiluncus species. A subtle but statistically significant inverse relationship between the number of Lactobacilli species and IL-1β level has been previously reported [25]. It is possible that this difference may be related to variations in the sensitivity of Gram staining versus culture as a method for enumerating the numbers of vaginal bacteria. The concept of a “threshold number” of bacteria required to induce a mucosal cytokine response has been previously described [26]. In colonization studies of the human urinary tract, it was shown that IL-6 levels were significantly elevated in urine with >10^5 Escherichia coli/mL, compared with the levels found in urine in which lower concentrations of bacteria were present. Interestingly, this bacterial concentration corresponded to the clinically defined level of “significant bacteriuria.” Our results suggest that a similar mechanism is present in the vagina—that is, that local cytokine production occurs when the concentration of facultative anaerobic gram-negative bacteria in the vagina reach concentrations associated with a Nugent-defined Gardnerella or Prevotella score of 4 (>30/high-power field from a vaginal smear).

Our results suggest 2 important conclusions with respect to the diagnosis and potential treatment of BV. First, given the likely role played by inflammatory cytokines in the serious

References
15. Hillier SL, Critchlow CW, Stevens CE, et al. Microbiological, epide-