Vaginal cytokines in normal pregnancy

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OBJECTIVE: The purpose of this study was to determine whether the vaginal cytokine concentration varies during the course of uncomplicated pregnancy.

STUDY DESIGN: Prenatal visits of healthy women to University Hospital Gasthuisberg, Leuven, Belgium were considered. Cytokine levels in vaginal washings from 30 unselected healthy women with uncomplicated pregnancies were monitored during pregnancy and compared with those from 62 nonpregnant healthy control subjects. Exclusion criteria included bacterial vaginosis, moderate or severe aerobic vaginitis, Trichomonas vaginalis, Candida vaginitis (wet mount or culture), gonorrhea, and Chlamydia. Interleukin-6, interleukin-8, interleukin-1β, interleukin-1–receptor antagonist, leukemia inhibitory factor, and tumor necrosis factor were measured. Nonparametric Kruskal-Wallis and Welch tests were used for univariate analysis, and the Spearman rank test was used for multivariate analysis.

RESULTS: Compared with concentrations in nonpregnant women, interleukin-1β concentrations were similar, but interleukin-1–receptor antagonist production was depressed throughout pregnancy. Vaginal interleukin-6 and interleukin-8 were less often discovered during pregnancy than outside pregnancy and dipped significantly in the middle trimester, to rise again to prepregnancy levels in the third trimester. Leukemia inhibitory factor was lower during the beginning of pregnancy (P = .038) but otherwise did not differ from nonpregnant values throughout pregnancy nor did tumor necrosis factor. Sexual activity could not explain these findings.

CONCLUSION: Vaginal cytokine levels, especially interleukin-1 receptor antagonist, from pregnant women may differ from nonpregnant values; some levels, such as interleukin-6 and interleukin-8, may fluctuate during normal pregnancy. These spontaneous variations during pregnancy must be taken into account when mucosal immunologic responses to infection of the lower genital tract are being studied. (Am J Obstet Gynecol 2003;189:1433-8.)

Key words: Cytokine, interleukin

The association of lower genital tract infection with an increased risk of preterm delivery and preterm rupture of the fetal membranes has stimulated great interest recently in the pathogenesis of such infection-related mechanisms. Most early studies show an increased rate of prematurity in women with bacterial vaginosis (BV), but later randomized trials and meta-analyses could not confirm unequivocally the efficiency of treatment strategies. Local immunologic responses to disturbances in the vaginal flora may explain the reason that some women have complications in pregnancy or whose condition fails to respond on treatment. It has been shown that the vaginal immunologic response to increasing grades of abnormal vaginal flora is dramatic and straightforward. Before engaging in the study of such host responses in pregnant women, however, it is crucial to know whether this mucosal immunologic response is influenced by the pregnancy itself. In this study, we investigated how the concentration of cytokines in the vagina depends on the stage of pregnancy.

Material and methods

Subjects. Internal review board approval was granted; after oral consent had been obtained, vaginal samples were taken from 30 healthy pregnant women at the obstetrics/gynecology clinic at Gasthuisberg University Hospital, Leuven, Belgium. Women were included only if they had not had signs or symptoms of vaginitis in the previous 2 months, had no history of recurrent vaginitis or pelvic inflammatory disease, and had not used antibiotics or any vaginal medication in the previous 2 months. Patients were asked to visit for sampling at the first pregnancy visit if less than 15 weeks of gestation and again at 28 to 32 weeks of gestation and at 36 to 38 weeks of gestation. No patients were delivered preterm or had abnormal or growth-restricted babies. Of the 90 samples that were taken during pregnancy, 12 samples were excluded because asymptomatic vaginal infection was
present as diagnosed by microscopy or vaginal culture, which left 78 samples for further analysis of the cytokines: 24 samples from the first trimester, 24 samples from the second trimester, and 30 samples from the third trimester. Sixty-two nonpregnant women who attended a parallel clinic and who matched the same exclusion criteria were used as control subjects.

An attempt was made to assess the influence of sperm or sexual activity on the concentration of vaginal cytokines by inquiry, at the moment of sampling, about the moment of the last sexual intercourse. A semiquantitative assessment of recent sexual activity was categorized as follows: last intercourse has taken place (1) < 24 hours ago, (2) 1 to 2 days ago, (3) 3 to 7 days ago, (4) 1 to 2 weeks ago, (5) >2 weeks and < 2 months ago, and (6) >2 months ago.

**Sampling and laboratory procedures.** On the three visits, an unmoistened sterile speculum was inserted before any other vaginal examination was performed. A sample of vaginal fluid was taken from the lateral vaginal vault with a wooden Ayre’s spatula and spread onto a glass slide. Saline solution was applied, and a coverslip was added for immediate microscopic evaluation. Vaginal pH was measured on the glass slide, by use of color strips with a pH range of 4.0 to 7.0 (Merck and Co, West Point, Pa). A standardized vaginal rinsing with 2 mL of 0.9% sodium chloride was made by flushing and reaspirating the fluid through a 0.5-mm wide, 6-cm long needle in the left, central, and right upper vaginal vaults. The rinses were centrifuged, frozen at –18°C and kept for later batch analysis, approximately 8 months after sampling. Both study samples and control samples were obtained during the same time period, kept together, and run together in batches. The supernatants measured for interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor (TNF), IL-1-receptor antagonist (IRA), and leukemia inhibitory factor (LIF).

**Immune assays of cytokines.** IL-1β, IL-6, IL-8, TNF, and IRA were determined with the high sensitivity enzyme-linked immunosorbent assay (ELISA) kits (Cistron). This sequential enzyme immunoassay is based on a monoclonal antibody that is specific for the respective human cytokines and has a dynamic range of 2 pg to 1000 pg/mL. LIF levels were determined with a monoclonal antibody–based, 2-step sandwich ELISA (ELISA kit catalog No. E04-18-1290; Eurogenetics;) with a dynamic range of 25 to 1000 pg/mL, where 25 pg/mL is the lower detection limit. For each of these immunologic methods, the vaginal fluid washings were diluted such that most of the resulting cytokine concentrations were located in the middle of the calibration curves for each cytokine.

**Vaginal cultures.** A cotton-tipped swab was taken from the posterior vault and immediately placed in Amies’ modified Stuart medium. After the ectocervix had been cleaned, an endocervical swab for *Chlamydia* culture on McCoy cells was allowed to soak for 20 seconds and was rotated three times in the endocervical canal. Vaginal and cervical cultures for *Gardnerella vaginalis* were grown on specific commercially available ampicillin-pretreated A3 media at 37°C for 18 hours. Other microorganisms (*Escherichia coli*, *Klebsiella* spp, *Acinetobacter* spp, *Staphylococcus* spp, *enterococci*, *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, and yeasts) were cultured on blood-chocolate agar.

**Exclusion of patients.** Women with redness of the vaginal wall, vaginal ulcerations, or offensive vaginal discharge were excluded. Samples were excluded from the cytokine analysis if any of the following diagnoses resulted: BV, moderate or severe aerobic vaginitis (AV), *Trichomonas vaginalis* (wet mount or culture), *Candida* vaginitis (either microscopy or culture positive), cervicitis (gonorrhoeae, *Chlamydia*, or other). BV was diagnosed as the presence of *Gardnerella, Mobiluncus* morphotypes, and/or clue cells on fresh wet mounts, when *G vaginalis* was cultured, or when a fishy smell was noted after the addition of 10% potassium hydroxide in water. The diagnosis of AV was based on a composite score, as described previously. If the AV score was above 5, the sample was excluded from further analysis.

**Statistical analysis.** Because the data did not follow normal distribution, mainly because many women did not express even some cytokines at all, the Kruskal-Wallis equation was used to compare the difference among all different groups, and the Welch equation was used for unequal variance to describe the difference between two groups. For numbers, the χ² test or the Fisher t test was used, where appropriate (low numbers).

**Results**

Except for the IL-1 receptor antagonist (Fig 1, Kruskal-Wallis equation, *P* < .0001), all cytokines from pregnant women had similar concentrations in vaginal fluid to those from nonpregnant control subjects (Table I, Kruskal-Wallis tests). IL-6 and IL-8, however, were produced less often during pregnancy than in nonpregnant women (Table I; odds ratio, 2.6 [95% CI, 1.2–5.3], respectively), and their production dropped to very low levels during the mid trimester of pregnancy (Welch test: *P* = .002 and *P* = .016, respectively) to raise levels back to normal prepregnancy levels during the final trimester (Welch test: *P* < .0001 and *P* = .02 respectively). LIF was produced less often during the first trimester (79% vs 95%) and, if produced, in lower concentrations than outside pregnancy (Table I; Welch test, *P* = .05).

Because recent or frequent sexual activity could have influenced the vaginal concentration that was caused by possible contamination with cytokines, the relationship of recorded recent sexual activity at the time of sampling with the vaginal cytokine content was also studied. The Kruskal-Wallis equation did not show any relationship of
sexual activity with IL-6, IL-8, LIF, IL-1β, or TNF. No significant correlation was found between IRA concentration and sexual activity, except in the group that had no or hardly any sexual activity (<1 in >2 months), in which group IRA was significantly higher ($P = .03$, Fig 2). Furthermore, in multivariate analysis, sexual activity was not linked with IRA concentration, but the state of pregnancy was (Table II).

**Comment**

The link between lower genital tract infections and preterm birth have been recognized for many years: gonorrhea, *Chlamydia*, primary syphilis, *Trichomonas*, vaginitis caused by group B streptococci, *E coli*, and BV all have been implicated in the pathogenesis of preterm birth, premature rupture of the membranes, and chorioamnionitis.5-10 Although high rates of
complications occur in some of these women despite treatment that should be adequate, there appear to be many other women who have the disorder and go through pregnancy without any problem. The various host responses that may be responsible partly for this have stimulated many investigators to study the differential response that a woman’s immune system may have to infectious challenge.

For instance, vaginal IL-1β concentrations increase as lactobacilli decrease. In studies of women during the first trimester of pregnancy or during labor, BV was also associated with IL-1β, but not with IL-6. There appear to be major differences in response between AV and BV. IL-1β increases in both conditions, but significantly more so in AV. Compared with women with normal flora, IL-6 remains unchanged in patients with BV but increases dramatically in patients with AV. Because IL-6 and IL-8 are markers for bacterial amnionitis and imminent term and preterm delivery and because IL-6, as a chemoattractant, is directly associated with increased prostaglandin

Table I. Mean concentrations of cytokines in vaginal rinsing fluids from 30 pregnant women and 62 nonpregnant control subjects with normal vaginal flora

<table>
<thead>
<tr>
<th>Vaginal fluid constituents (n)</th>
<th>Trimester of pregnancy</th>
<th>Statistical difference (P&lt;5 value) of non-parametric tests: †</th>
<th>a) Welch t test</th>
<th>b) Kruskal-Wallis analysis</th>
<th>c) χ² or Fisher T test (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0: Not pregnant (n = 62)</td>
<td>I: 14 Gestational weeks (n = 24)</td>
<td>II: 14-28 Gestational weeks (n = 24)</td>
<td>III: &gt;28 gestational weeks (n = 30)</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/mL)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>24 (39%)</td>
<td>4 (17%)</td>
<td>4 (17%)</td>
<td>5 (17%)</td>
<td></td>
</tr>
<tr>
<td>IL-8 (pg/mL)*</td>
<td>3.2 (6.8)</td>
<td>2.3 (5.4)</td>
<td>0.8 (1.9)</td>
<td>5.7 (11)</td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>26 (42%)</td>
<td>4 (17%)</td>
<td>3 (13%)</td>
<td>10 (33%)</td>
<td></td>
</tr>
<tr>
<td>IL-1β (pg/mL)*</td>
<td>149 (319)</td>
<td>318 (635)</td>
<td>62 (100)</td>
<td>94 (211)</td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>33 (85%)</td>
<td>18 (78%)</td>
<td>16 (70%)</td>
<td>29 (69%)</td>
<td></td>
</tr>
<tr>
<td>IRA (ng/mL)*</td>
<td>113 (74)</td>
<td>41 (38)</td>
<td>64 (62)</td>
<td>67 (73)</td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>58 (98%)</td>
<td>20 (95%)</td>
<td>19 (90%)</td>
<td>27 (96%)</td>
<td></td>
</tr>
<tr>
<td>LIF (pg/mL)*</td>
<td>65.6 (170)</td>
<td>19 (14)</td>
<td>47.5 (137)</td>
<td>29 (47)</td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>58 (95%)</td>
<td>19 (79%)</td>
<td>23 (96%)</td>
<td>27 (97%)</td>
<td></td>
</tr>
<tr>
<td>TNF (pg/mL)*</td>
<td>6.5 (15)</td>
<td>4.9 (9.9)</td>
<td>7.8 (11)</td>
<td>9.8 (17)</td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>11 (22%)</td>
<td>5 (21%)</td>
<td>8 (36%)</td>
<td>8 (30%)</td>
<td></td>
</tr>
</tbody>
</table>

*Only significant relations are shown.
†Numbers in brackets represent standard deviation.
‡Only 39 samples were available for this analysis.
§Only 58 samples were available for this analysis.

Table II. Spearman rank analysis of the variable IRA as a function of age, parity, level of recent sexual activity, and state of pregnancy

<table>
<thead>
<tr>
<th>IRA</th>
<th>Regression coefficient</th>
<th>T value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.6</td>
<td>−0.65</td>
<td>.52</td>
</tr>
<tr>
<td>Parity</td>
<td>−3.6</td>
<td>−0.53</td>
<td>.61</td>
</tr>
<tr>
<td>Level of recent sexual activity</td>
<td>2.2</td>
<td>0.41</td>
<td>.68</td>
</tr>
<tr>
<td>Gestational status (non pregnant, 1st, 2nd, 3rd trimester of pregnancy)</td>
<td>−46</td>
<td>−2.9</td>
<td>.0055</td>
</tr>
</tbody>
</table>

Candida, bacterial vaginosis, Trichomonas vaginalis, aerobic vaginitis, gonorrhea, and Chlamydia were negative in all samples.

Fig 2. IRA compared with recent sexual activity. 1, Intercourse <24 hours ago; 2, 1 to 2 days ago; 3, 3 to 7 days ago; 4, 1 to 2 weeks ago; 5, >2 weeks ago or <2 months ago; 6, >2 months ago. The Kruskal-Wallis difference between the groups (P = .03) is completely due to the group having hardly any or no sexual activity.
production and delivery, such findings may have implications in the management of pregnancy.

However, to study the effect of these or other infections during pregnancy, let alone the effect of treatment, the influence of pregnancy itself on these immunologic responses must be clarified. We present results for a wide array of cytokines that may be involved in infectious disease–related pathologic conditions during pregnancy. Both pregnant cases and control subjects were selected according to the most stringent criteria of having no infection, either in the past or at the time of sampling, either symptomatic or asymptomatic. All pregnancies evolved normally, and babies were born spontaneously with normal birth weights.

The proinflammatory cytokines IL-6 and IL-8, mostly linked to imminent term or preterm delivery, were severely suppressed during the mid trimester. IL-6 and the more pronounced IL-8 levels resurged to increased concentrations near term, but IL-1β levels remained low. IL-1β levels decreased, although not significantly, as pregnancy progressed; not only the mean concentrations were lower, but also the number of women with IL-1β presence declined gradually. At the same time, IRA is severely suppressed throughout pregnancy, thereby potentiating the effect of the available IL-1β. Once the women were pregnant, the IRA concentration remained remarkably stable during the entire course of pregnancy, again allowing IL-1β to exert its effects. IL-1β was shown previously to correlate with lactobacillary grades, having the highest concentration when the lactobacillary morphotypes are displaced completely by other morphotypes (lactobacillary grade III) and lowest when lactobacilli are abundant.3 During pregnancy, the likelihood of having BV decreases toward the end of pregnancy, while the healthy, lactobacilli-dominant flora increases with increasing gestational age.17 Hence, the production of vaginal IL-1β, and its ratio to its IRA, seem to reflect the relative number of functional lactobacilli inversely as a prime vaginal defense mechanism. An unexpected increase may indicate that lactobacilli are decreasing at the advantage of other microorganisms. Dowgrading IL-1β production, and hence the IL-1β/IRA ratio, will also reduce the stimulation of T H 2 lymphocytes, which leads to a sharp drop in the production of both proinflammatory cytokines IL-6 and IL-8, as was seen in the second trimester. The reason that these cytokines rise again to prepregnancy levels at the very end of pregnancy seems to be related to the imminent birth process that is in preparation. Just before delivery, increased levels of these cytokines have been detected in amniotic fluid in women with intact fetal membranes and in the absence of chorioamnionitis.18 In the case of intrauterine bacterial infection, however, the increase is more pronounced. Therefore, if high levels of IL-6 and IL-8 were to be encountered in the second trimester, this may indicate an increased risk of infection-related preterm birth or intrauterine infection.

LIF is involved in the pharmacologic condition of ovulation and fertility and is known to inhibit the proliferation of leukocytes. Interestingly, its concentration in the vagina was 3-fold depressed during the beginning of pregnancy. In another study, in the presence of abnormal vaginal flora, LIF was dramatically increased in patients with AV, but not in patients with anaerobic BV.19 This may be related to the fact that, in uncomplicated full-blown BV, no leukocytes are present; so there is no reason to elicit an antileukocyte response. In AV, on the other hand, the presence of leukocytes and the so-called “toxic” leukocytes indicates the severity of the pathologic condition, and LIF concentrations are found to be increased. LIF concentrations were linked directly to the number of leukocytes that are present, even in the severest form of infection when leukocytes were overly abundant and the IL-1β/IRA ratio was reversed.3

We conclude that profound changes are prevalent in the concentrations of vaginal cytokines, depending not only on the state of pregnancy but also, to some extent, on the gestational age. These changes have to be taken into account when studies of the influence of bacterial microflora on pregnancy are undertaken. Further studies to differentiate the effects of BV and AV on the outcome of pregnancy are needed urgently, because they may help answer the question of why some studies found no association between BV or its treatment and pregnancy outcome, although others found that restoring the flora to normal prevented preterm birth.

REFERENCES


