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Subtle perturbations of genital microflora alter mucosal immunity among low-risk pregnant women

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Abstract

Objective—Alteration in the vaginal flora has been associated with adverse pregnancy outcomes. The objective of this study was to examine the impact of changes in individual microflora on genital immunity among low-risk pregnant women in early pregnancy.

Design—Cross-sectional study.

Setting—Large, tertiary care, academic hospital clinic.

Population—Low-risk women were enrolled prior to 14 weeks' gestation.

Methods—Women were included if they had no medical or previous obstetrical complications, were non-smokers, had no sexually transmitted infections and no intercourse in the last 48 hours. Consenting women underwent speculum examination for collection of vaginal culture and Dacron swabs for cytokine analysis. Semi-quantitative vaginal cultures were performed in a reference laboratory.

Main Outcome Measures—Concentrations of immune mediators were compared in the presence of various organisms. Concentrations were converted to multiples of the median to standardize the values of each mediator. Regression analyses were performed to control for race.

Results—We enrolled 47 women. The frequencies of genital microorganisms were: H₂O₂-producing lactobacilli (70%), *Ureaplasma urealyticum* (66%), *Gardnerella vaginalis* (45%), anaerobic non-pigmented Gram-negative rods (ANPGNR, 40%), anaerobic pigmented Gram-negative rods (APGNR, 17%). After adjusting for race and body mass index, interleukin-1 β , interferon- γ , tumor necrosis factor- α and granulocyte macrophage-colony stimulating factor were increased in the presence of *G. vaginalis*, ANPGNR, and APGNR. There was no consistent impact on the other markers of immune activation.

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Conclusion—The presence of individual species impacts genital immunity among low-risk pregnant women. Perturbations in genital immunity could partially explain heterogeneity in adverse pregnancy outcomes.

Keywords

Genital immunity; vaginal flora; cytokines

Introduction

According to data reported by the March of Dimes, preterm birth increased over a 10-year period from 1996 to 2006 (1). Infant mortality resulting from preterm birth did not decrease over the period from 1996 to 2005. Preterm birth is the second leading cause of neonatal mortality after congenital anomalies (1). Although there has been significant attention to the problem of preterm birth, there has not been much progress made in decreasing its occurrence. Despite this, the initiating mechanism of the cascade of events leading to spontaneous preterm birth remains poorly understood.

A key to understanding the mechanism of preterm birth may involve characterizing the immune state of the female lower genital tract. Cytokines and chemokines are proteins that function once the innate immune response is activated. Chemokines are proteins that signal the recruitment of inflammatory cells to a site of insult. Cytokines generate the effects of immune inflammatory cells. Alterations in cytokine profiles have been clearly associated with adverse pregnancy outcomes (2–4).

Many studies have examined the relation between a variety of pathogenic states and cytokine and chemokine expression within the lower genital tract or the amniotic cavity (2–8). Whereas the association between the host inflammatory milieu and spontaneous preterm birth has been documented, there has been little progress toward identification of potential therapeutic targets. Little work has connected inciting stimuli for an inflammatory response and the cascade of events that lead to preterm birth. An area of vaginal immunology that is poorly characterized in pregnancy is the component of innate immunity that provides the first line of defense at the epithelium of mucosal surfaces. This includes the normal vaginal microbial flora as well as the antimicrobial peptides that coat these surfaces. Although vaginal flora is generally known to contain a variety of organisms, there is sparse literature regarding changes in the vaginal ecosystem in normal pregnancy (9). The syndrome of bacterial vaginosis (BV) has been extensively studied and found to be associated with an increased risk of preterm birth (odds ratio (OR) 2.19) in a large meta-analysis (10). Most women with BV do not experience preterm birth, and treatment of BV has not been consistently shown to improve outcomes (11–17). This heterogeneity in clinical presentation and outcomes suggests that the contribution of BV to preterm birth is incompletely understood.

Most investigations have focused on the presence or absence of the diagnosis of BV, the definition of which varies between studies (2,11,18,19). A more recent report has found an association, however, with other alterations in vaginal flora and preterm birth. Carey and Klebanoff performed an analysis of data from the Vaginal Infections and Prematurity study

and found that a change in vaginal flora to heavy growth of *Escherichia coli* or *Klebsiella pneumoniae* was associated with an increased risk of preterm birth (20). The focus of this study was to examine whether subtle perturbations in vaginal flora are associated with disruptions in the maternal host inflammatory response.

Material and methods

First trimester vaginal flora data from an ongoing prospective observational cohort study were analyzed. Low-risk women presenting for routine prenatal care at a large, tertiary care institution between July 2007 and August 2009 were enrolled, and vaginal and serum samples collected. Women were approached if they were less than 14 weeks' gestation by best obstetrical estimate based on last menstrual period and the earliest ultrasound dates at the time of their visit to their prenatal care provider. Women were asked to avoid the use of vaginal products and intercourse for 48 hours prior to the study examination. To enroll a cohort at low risk for baseline alteration in inflammatory mediators, such as cytokines, there were many exclusion criteria. Women were not included if they met any of the following exclusion criteria: acute systemic illness, chronic illnesses requiring medication, systemic steroid use in the past 3 months, immunization in the past month, active alcohol, drug, or tobacco use, immunocompromised state, known active infection due to *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, bacterial vaginosis by Amsel's criteria (21), or clinically apparent herpes simplex virus. Women were also excluded if they reported symptomatic vaginal discharge, urinary tract infection, antibiotic use within the last month, chronic hypertension, preexisting diabetes mellitus, prior preterm birth 16 0/7–36 0/7 weeks, current or planned cerclage suture, history of preeclampsia occurring at <36 weeks' gestation, known multiple gestation, or history of thromboembolic disease.

Enrolled women provided written, informed consent and the study was approved by the Women & Infant's Hospital of Brown University Institutional Review Board. Medical, social, and demographic information was collected from each subject. Serum was collected by venipuncture and stored at –80°C for evaluation of systemic inflammation via a highly sensitive C-reactive protein (hsCRP) assay. A pelvic speculum examination was performed for collection of vaginal culture, vaginal secretory leukocyte protease inhibitor (SLPI), and cervical cytokines and chemokines. The vaginal cultures were collected using a Dacron-tipped swab and placed in Port-a-cul transport gel (Becton-Dickinson, Sparks, MD, USA). The swabs were shipped overnight to Pittsburgh, Pennsylvania, where the cultures were performed in a reference laboratory. The culture was performed in a standardized fashion during which swabs were removed from the transport gel and inoculated onto Columbia agar supplemented with 5% sheep blood, two plates of human bilayer Tween (HBT) agar, Rogosa agar, A-8 agar, Ureaplasma broth, and one plate of prerduced laked blood kanamycin agar. The Columbia agar, one set of HBT agar plates, the A-8 agar plate, and the Ureaplasma broth were incubated at 36°C in 5–7% CO₂ for a minimum of 48 hours. The remaining plates were incubated within an anaerobic glove box at 36°C for a minimum of 5 days. Biochemical tests were used to identify *Enterococcus*, *E. coli*, and Group B *Streptococcus* (GBS), *Mycoplasma*, *Ureaplasma*, *Gardnerella vaginalis*, *Lactobacillus* and anaerobic Gram-negative rods were identified by their characteristic colony morphology on selective media and Gram stain morphology. Pigmented organisms have a black appearance and include

organisms such as various *Porphyromonas* species. The non-pigmented Gram-negative rods commonly represent various *Prevotella* species. *Lactobacilli* were tested for production of H₂O₂ in a qualitative assay on a tetramethylbenzidine agar plate. After 2 days of incubation in an anaerobic glove box at 37°C, the tetramethylbenzidine agar plates were exposed to ambient air. The H₂O₂ that is formed reacts with horseradish peroxidase in the agar, causing the colonies to turn a characteristic blue color, identifying hydrogen peroxide-producing *Lactobacilli*. Other species of *Lactobacilli* are non- H₂O₂-producing species.

Cervical cytokine swabs were stored in 100µl of phosphate-buffered saline (PBS) at –80°C until processing. The cytokine and chemokine concentrations were assessed using the Luminex® multiplex assay in duplicate (Luminex Corp., Austin, TX, USA). The sample was diluted and mixed with Beadlyte® multi-cytokine beads (Upstate USA Inc., Charlottesville, VA, USA). The sample was then mixed with anti-human multi-cytokine reporter and fluorescence-labeled. The Luminex® laser then detected and quantified the analyte present on the beads. The Luminex® multi-cytokine bead method has been previously validated and shown to have excellent correlation with standard ELISA methods (22). The lower limit of detection for all cytokines was 2.3 pg/ml. The coefficient of variation (CV) was 10% on average. The cytokines and runs that were above 10%CV were predominantly those that were at the low end of the scale. Vaginal SLPI swabs were stored dry at –80°C until processing. The SLPI assay is a commercially available standard ELISA, Quantikine Human SLPI Immunoassay kits (R&D Systems Inc, Minneapolis, MN, USA), and was performed according to manufacturer's instructions.

The hsCRP assay is a simple sandwich ELISA adapted from that of Erhardt et al. (23). The plates were coated in diluted rabbit polyclonal anti-CRP antibody (Dako, Glostrup, Denmark) and incubated. Serum samples were diluted 1:1000–1:10,000 to fit a standard curve, and incubated in the antibody-coated wells. The samples were then incubated with the secondary antibody, diluted polyclonal HRP-conjugated rabbit anti-CRP antibody (Dako). After washing, TMB peroxidase substrate (Bio-Rad, Hercules, CA, USA) was added for color reagent.

Sample size was calculated using values of SLPI because there are published values of SLPI in normal pregnancy. Draper et al. reported that concentration of SLPI at 14 weeks was 641,000 pg/ml, with a standard deviation of approximately 150,000 pg/ml (24). The sample size needed to detect a 30% change in baseline SLPI concentrations was 20 women with a 90% power and alpha=0.01. This study was over-enrolled with a total of 40 women to account for planned multiple comparisons. For the analysis, concentrations of cytokines, SLPI and hsCRP were converted to multiples of median (MOM). This method facilitates comparison of the different markers by standardizing values to one scale. The lower limit of detection was used for analysis in samples falling below this value. Converted MOMs of the various mediators were compared in the presence of growth of various organisms. MOMs were calculated by dividing each marker by the median value among those with no growth of the organism. The van Elteren test, a stratified version of the Wilcoxon rank sum test, was used to adjust for race and body mass index (BMI). We chose to adjust for race due to the association of altered flora and changes in inflammatory mediators with racial group. We

adjusted for BMI due to the association of changes in inflammatory mediators, particularly CRP, with increased BMI.

Results

In all, 47 pregnant women were enrolled in the study. Three were missing immune marker data and one was missing vaginal flora culture results. The median gestational age at enrollment was 12 weeks (range 8–14). Most of the women had at least some college education and were employed. The demographic characteristics of the cohort are displayed in Table 1. Six women had a history of genital herpes but did not have symptoms at the time of the examination. Interestingly, this cohort of seemingly low-risk pregnant women was colonized with organisms that are frequently considered to be pathogenic. The frequency of vaginal colonization by selected organisms is shown in Table 2. Nearly a third had no growth of H₂O₂-producing lactobacilli. Nearly half were colonized with *G. vaginalis*. There was also a clearly detectable rate of colonization with aerobic and anaerobic Gram-negative rods, *Enterococcus*, *Staphylococcus aureus*, and *E. coli*. Nearly 70% were colonized with *Ureaplasma* spp. Other organisms that were detected included *Mycoplasma* and *Candida* spp.

None of the women had a clinical diagnosis of bacterial vaginosis. The median vaginal pH was 4.4 (4.0–6.1). The association with changes in various inflammatory markers was determined for each of the target organisms identified. The adjusted MOMs for each marker in the presence of certain organisms are presented in Table 3. After adjusting for race and BMI, significant increases were consistently observed for interleukin (IL)-1 β , interferon (IFN)- γ , granulocyte macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF)- α associated with colonization by *G. vaginalis*, or anaerobic Gram-negative rods, both pigmented and non-pigmented. There were no changes in the concentrations of vaginal SLPI or in the systemic marker of inflammation hsCRP associated with colonization by any of the bacteria.

None of the other bacteria was consistently associated with significant changes in multiple inflammatory marker concentrations. Colonization with *Ureaplasma* spp. was associated with increased IL-10 concentrations, adjusted $p=0.02$, but not with changes in other mediators. Absence of growth of H₂O₂-producing lactobacilli, one of the hallmark changes associated with bacterial vaginosis, did not impact on local or systemic inflammatory markers. The presence of *Ureaplasma* or *Mycoplasma* spp. did not alter these markers.

Discussion

We found that vaginal colonization with certain organisms was associated with changes in the concentration of multiple local immune mediators. The presence of *G. vaginalis* and anaerobic pigmented and non-pigmented Gram-negative rods were all associated with increases in proinflammatory cytokines, the chemokine GM-CSF and, in the case of Gram-negative rods, the anti-inflammatory cytokine IL-10. We did not, however, observe evidence of systemic inflammation as measured by hsCRP or disruption of the protective antimicrobial peptide SLPI in the presence of such organisms. We also did not see evidence

of significant local or systemic immune changes in the presence of certain organisms that have previously been debated in terms of risk of adverse pregnancy outcomes, such as *Ureaplasma* spp. and *Mycoplasma* spp. (25,26). The frequency of identification of these organisms, while higher than in some earlier reports (26), was not unexpected, as this rate of colonization has been previously reported in pregnancy (25). Our study was somewhat unique, however, in that this was a very low-risk group of pregnant women.

The study revealed that a large proportion of low-risk pregnant women are colonized with a vaginal flora that in the past was thought to be associated with adverse outcomes. There was a high prevalence of *G. vaginalis*, *Ureaplasma* spp., and *Mycoplasma* spp. There was also colonization with known potential pathogens such as *S. aureus* and *E. coli*. Colonization with these organisms during pregnancy has been reported in prior large studies (20). Donders et al. reported an entity in non-pregnant women that they termed ‘aerobic vaginitis’, in which they reported that increased IL-1 β concentrations were associated with certain microscopy abnormalities that were distinct from BV (27). This work supports the notion that the abnormalities of vaginal flora seen in BV likely represent a spectrum of flora disruption.

The study was limited by the relatively small size of the cohort to evaluate the true prevalence of various organisms. While it would be ideal to have a larger sample for evaluation of the prevalence of many vaginal microorganisms, we took care to include a very low-risk cohort to avoid alteration in the immune profile that might be attributed to something other than the study conditions. We used a selected panel of organisms and did not examine the prevalence of viral organisms and did not perform Gram staining for evaluation of bacterial vaginosis. This panel was selected to reflect common organisms cultured from the vagina as well as potential vaginal pathogens. Women with symptomatic vaginal discharge were excluded. The rare finding of significant clue cells in only three women suggests that the women in the cohort were unlikely to have asymptomatic BV. This study was designed to examine low-risk women only and therefore a high risk comparison group was not included.

Strengths of the study include its prospective nature and careful inclusion and exclusion criteria. The sample was carefully selected to avoid including women with conditions that might alter the vaginal or systemic immune state. We believe this diminished the impact of potential confounding variables on the study conditions. The study also avoided difficulties in organism recovery frequently seen in clinical microbiology laboratories by utilizing a reference research laboratory for vaginal culture techniques. This allowed for culture of organisms that are considered difficult to culture, such as *Ureaplasma* and *Mycoplasma* spp.

The finding of immune activation seen in association with individual microflora, even in the absence of meeting diagnostic criteria for bacterial vaginosis, is unique and has implications for understanding the pathogenesis of vaginal flora disruption and its association with clinical outcomes. Meta-analyses have described associations between bacterial vaginosis and HIV acquisition (28) and preterm birth (10). These clear statistical associations, however, are not causal. The inflammatory cascade that presumably is created by the presence of bacterial vaginosis (29) and then leads to the dynamic changes of labor are as

yet undefined. Mattsby-Baltzer et al. reported that cervicovaginal IL-1 β was increased in the first trimester among women with clinical diagnosis of BV by two of the criteria of Amsel et al., but they did not observe increased IL-6 or TNF- α as we did with individual microflora in this study (30). Interestingly, that study did find capability of cytokine activation from cervical mucous endotoxin.

The next logical step to determining the impact of this immune activation as a result of subtle changes of microflora is assessing whether therapy would improve such changes. Yudin et al. reported in a randomized trial that among women with a diagnosis of BV by Gram stain and clinical criteria, therapy resulted in decrease of cervical IL-1 β , IL-6, and IL-8 concentrations only in the women who established normal flora following therapy (29). It is unclear whether therapy for more subtle changes in flora would have a similar impact. Future studies focused on subtle changes in microflora are warranted.

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Abbreviations

BV	bacterial vaginosis
ANPGNR	anaerobic non-pigmented Gram-negative rods
APGNR	anaerobic pigmented Gram-negative rods
OR	odds ratio
hsCRP	C-reactive protein
SLPI	vaginal secretory leukocyte protease inhibitor
MOM	multiple of the median
IL	interleukin
IFN	interferon
GM-CSF	granulocyte macrophage-colony stimulating factor
TNF	tumor necrosis factor

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Table 1

Demographic characteristics of the cohort.

Characteristic	Frequency n=47
Age, median (range)	25 (18–35)
Gestational age, median (range)	12 (8–16)
Race, n (%)	
Latina	21
Asian	2
Black	6
White	26
BMI, * median (range)	25 (16–52)
Education level *, n (%)	
Some college or greater	30
Employed, n (%)	34
Insurance, * n (%)	
Uninsured	7
Federal	20
Private	17
Other	2

* For those for whom the information was available, one missing.

Table 2

Frequency of colonization with various organisms.

Organism (n=47)	Frequency n (%)
H ₂ O ₂ -producing lactobacilli	33
Non-H ₂ O ₂ -producing lactobacilli	20
<i>Gardnerella vaginalis</i>	21
Anaerobic non-pigmented Gram-negative rods	19
Anaerobic pigmented Gram-negative rods	8
Enterococcus	6
<i>Ureaplasma</i> spp.	31
<i>Mycoplasma</i> spp.	9
<i>Staphylococcus aureus</i>	4
<i>Escherichia coli</i>	1

H₂O₂, hydrogen peroxide; spp, species.

Table 3

Immune activation by microflora.

Marker	<i>Gardnerella vaginalis</i>		Anaerobic non-pigmented Gram-negative rods		Anaerobic pigmented Gram-negative rods	
	Median MOM [*] (Range)	<i>p</i> [†]	Median MOM [*] (Range)	<i>p</i> [†]	Median MOM [*] (Range)	<i>p</i> [†]
IL-1 β (n=43)	7.9 (0.7–401.3)	0.002	6.7 (0.04–317.1)	0.03	9.9 (2.5–60.7)	0.01
IL-6 (n=40)	2.1 (0.03–7.1)	0.1	1.8 (0.02–6.4)	0.5	2.8 (0.6–6.4)	0.04
IFN- γ (n=43)	21.7 (1.0–107.7)	0.0004	1.8 (0.1–10.3)	0.05	2.0 (0.06–6.6)	0.02
IL-10 (n=43)	12.0 (1.0–77.8)	0.2	18.3 (1.0–77.8)	0.03	31.8 (1.0–77.8)	0.004
GM-CSF (n=43)	19.0 (1.0–265.8)	0.008	16.5 (1.0–265.8)	0.003	62.2 (2.9–265.8)	0.002
TNF- α (n=43)	6.7 (1.0–91.3)	0.01	5.7 (1.0–91.3)	0.02	6.7 (1.0–91.3)	0.02
SLPI (n=46)	1.6 (0.05–15.6)	0.9	1.1 (0.1–11.6)	0.8	2.0 (0.3–12.5)	0.6
hsCRP (n=46)	0.9 (0.08–4.4)	0.9	0.8 (0.1–4.1)	0.6	1.1 (0.3–2.4)	0.7

MOM, multiples of the median; IL, interleukin; IFN, interferon; GM-CSF, granulocyte macrophage colony stimulating factor; SLPI, secretory leukocyte protease inhibitor; hsCRP, highly sensitive C-reactive protein.

* MOMs for organism growth were calculated as the marker value divided by the median value for no growth.

[†] Adjusted for race (White vs. non-White) and body mass index (25 vs. <25).