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Interrelationships of interleukin-8 with interleukin-1β and neutrophils in vaginal fluid of healthy and bacterial vaginosis positive women

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Vaginal innate immunity in response to microbial perturbation is still poorly understood and could be crucial for protection from adverse outcomes. We investigated the relationship between interleukin (IL)-8, IL-1β and neutrophils in vaginal fluid obtained from 60 healthy women and 51 women who were bacterial vaginosis (BV) positive. Concentrations of IL-8 and IL-1β were highly correlated with counts of neutrophils in vaginal fluid of the entire population examined (111 subjects). Vaginal IL-1β concentrations were significantly higher ($P < 0.001$) in BV positive women. There was no significant difference in IL-8 levels or number of neutrophils between healthy controls and BV positive women. None of the healthy controls with high neutrophil counts (≥75th percentile, 14 average count per field) had high concentrations of IL-1β (≥75th percentile, 220 pg/ml), whereas 84% of BV positive women with high neutrophil counts had high IL-1β concentrations ($P < 0.001$). On the contrary, no difference in the percentage of subjects with elevated concentrations of IL-8 (≥75th percentile, 2842 pg/ml) was found between healthy and BV positive women with high numbers of neutrophils (55.5% of healthy versus 53% of BV positive women). Our findings show that BV causes a large increase in IL-1β concentrations which is not paralleled by an increase in IL-8 concentrations in vaginal fluid, suggesting that BV-associated factors more specifically dampen IL-8 rather than IL-1β. The lack of an increase in IL-8 may explain the absence of an increase in neutrophil numbers in most women exposed to abnormal vaginal colonization (BV).

Key words: bacterial vaginosis/interleukin-1β/interleukin-8/mucosal innate immunity/vaginal leukocytes

Introduction

Mucosal immune system activation, especially innate immunity, represents a critical response against microorganisms colonizing the reproductive tract that could prevent severe complications associated with abnormal vaginal flora (Fidel *et al*., 1997; Cauci *et al*., 1998, 1999, 2002a; Donders *et al*., 2000). Neutrophil recruitment and activation is considered the main innate immune response against microbial and viral infections of the vaginal mucosa (Milligan *et al*., 2001). Many aspects of vaginal innate immunity are still to be disclosed. Bacterial vaginosis (BV) is the most prevalent vaginal disorder in non-pregnant and pregnant women (Hillier *et al*., 1995; Cauci *et al*., 2002b). It is associated with several adverse outcomes, including increased susceptibility to human immunodeficiency virus (HIV) infection (Sewankambo *et al*., 1997), upper genital tract infections (Sweet *et al*., 2000), endometritis (Ness *et al*., 2001), post-surgical infections (Guaschino *et al*., 2002), urinary tract infections (Hillebrand *et al*., 2002), and adverse pregnancy outcomes (miscarriage, preterm delivery and low birthweight) (Hillier *et al*., 1995; McGregor and French, 2000). BV is a complex polymicrobial disorder characterized by a decreased lactobacilli flora and a largely increased colonization of several facultative and strictly anaerobic microorganisms, mainly *Gardnerella vaginalis*, *Prevotella* spp., *Bacteroides* spp., *Mobiluncus* spp., gram-positive cocci, and *Mycoplasmas* (Eschenbach, 1993). BV-associated alterations of the vaginal mucosal system are partially understood. However, it is not known why most women with BV show no inflammatory signs, whereas other vaginal infections such as Trichomoniasis, or Candidiasis, cause major inflammatory symptoms and leukocyte accumulation.

Interleukin (IL)-8 is a potent chemotactic and activating factor for neutrophils that has been detected in vaginal fluid of women with BV and other vaginal infections (Shaio *et al*., 1995; Filler *et al*., 1996; Wennerholm *et al*., 1998). IL-8 is a pleiotropic cytokine with several functions; it is produced by many different kinds of cells including neutrophils, monocytes, macrophages, endothelial and epithelial cells in response to different stimuli. IL-8 has many roles in the reproductive tract (Runesson *et al*., 1996; Khatun *et al*., 1999; Sennstrom *et al*., 2000). Several innate factors such as IL-1, neutrophil elastase and matrix metalloproteinase-9 stimulate production or enhance activity of IL-8 (Denison *et al*., 2000; Elliott *et al*., 2001). A recent study showed that impairment of IL-8 induction in women with BV is associated with low levels of vaginal immunoglobulin

(Ig)A against haemolysin produced by *G. vaginalis* (anti-Gvh IgA), low number of leukocytes, and high microbial hydrolytic enzyme activities (Cauci *et al*., 2002a).

IL-1β is considered a key mediator to contrast microorganisms. In fact IL-1β is a master cytokine inducing several chemokines, especially IL-8. IL-1β rises in response to several stimuli including a vast array of pathogens (Dinarello, 1996). Increased levels of IL-1β in cervicovaginal secretions have been associated with HIV type 1 infection (Spear *et al*., 1998), and BV (Sturm-Ramirez *et al*., 2000). Recent findings have demonstrated that low concentrations of IL-1β were associated with low levels of anti-Gvh IgA in vaginal fluid of women with BV (Cauci *et al*., 2002c).

The mechanisms that lead to an appropriate host response effective in controlling and eradicating vaginal infection needs to be investigated. Identification of different profiles among women with BV, especially by means of vaginal indicators, could differentiate patients at high risk of BV adverse outcomes and/or those who need treatment. The present study aimed to assess the relationship between IL-8, IL-1β and neutrophils in vaginal fluid of healthy and BV positive women.

Materials and methods

Women were recruited during routine gynaecological examinations to perform Papanicolau examination in three clinics located in Udine, Trieste and Bologna, Italy, from April 2001 to May 2002. All women were enrolled after informed consent according to the Institutional Ethics Committees. Inclusion criteria were: white, fertile age, non-pregnant, pre-menopausal women, without severe medical illnesses, declaring no sexual intercourse or any vaginal practices in the last 3 days, and no antibiotic use in the last 2 weeks. Exclusion criteria were: presence of bleeding or major lower vaginal tract inflammatory signs, yeast vaginitis (by clinical signs and by detection of yeast hyphae or spores in the vaginal smear), *Trichomonas vaginalis* (evaluated on a wet smear and/or Pap smear exam), *Neisseria gonorrhoeae* (based on clinical criteria confirmed by swab culture on Thayer-Martin medium, Oxoid, Milano, Italy), and *Chlamydia trachomatis* (by ligase chain reaction, LCx; Abbott Diagnostics, Rome, Italy). Enrolled women were aged 18–49 years. Cases and controls were not statistically different in contraceptive use, and in menstrual cycle phase. Women with BV were recruited when all four Amsel criteria were positive (Amsel *et al*., 1983), vaginal pH range was 4.7–6.5 (by pH paper strip; Merck, Bologna, Italy). Healthy controls had no signs of any vaginal pathological condition at least in the last 3 months and had vaginal pH ⩽4.5 at visit. All subjects had a normal PAP examination.

Vaginal specimens were collected as previously described (Cauci *et al*., 1996). Vaginal fluid was retrieved by lavage using 10 ml of sterile saline. Gram-stained smear evaluation was performed according to Nugent (1991). The group of healthy controls had Nugent score 0 and 1. The women with BV were all positive for clue cells (typical epithelial cells surrounded by adherent bacteria) on the Gram-stained smear and had Nugent score from 5 to 10. The number of neutrophils was evaluated on the Gram stained smear by averaging the counts of five different fields under oil immersion (×1000 magnification) (Hitti *et al*., 2001; Cauci *et al*., 2002a). More than 30 neutrophils per field were arbitrarily ranked as 40 for statistical calculations.

IL-8 and IL-1β were quantified in vaginal fluid by enzyme-linked immunosorbent assay, and measurements were performed according to the manufacturer's instructions (CLB, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). The intra- and inter-assay coefficients of variation for the IL-8 test were <5% and <10%, and for the IL-1β assay were <10% and <10% respectively. The lower detection limit for human IL-8 was 8 pg/ml and for IL-1β it was 1 pg/ml; a zero value was assigned to samples below these limits, one woman (a BV positive patient) had undetectable IL-8, and seven women (all healthy subjects) had undetectable IL-1β levels. Values >75th percentile were considered high levels of IL and neutrophils. All measurements were performed in duplicate.

Statistical analysis

The Spearman rank correlation was used to examine the correlations between immune factor levels in vaginal fluid. Two-tailed significance of Spearman rho coefficient (r_s) was reported. Cytokine concentrations were not normally distributed, thus the Mann–Whitney *U*-test was used to compare the concentrations of vaginal factors between healthy and BV positive women or between BV subgroups. Difference of proportions was assessed by χ^2-test. $P < 0.05$ was considered statistically significant. The software package SPSS (Statistical Package for Social Sciences) was used for data analyses.

Results

Table I shows the comparison of the median values of neutrophil counts, IL-8 and IL-1β concentrations in vaginal fluid specimens of healthy and BV positive women. Neutrophils and IL-8 concentrations of cases and controls were not significantly different ($P = 0.171$ and $P = 0.189$). By contrast, BV positive women showed a 19-fold increase in IL-1β concentrations (median value of 211 pg/ml, versus 11 pg/ml of healthy controls, $P < 0.001$).

Neutrophil counts were strongly correlated (using the Spearman rank correlation test) with IL-8 and IL-1β concentrations in all 111 enrolled women ($r_s = 0.596$, $P < 0.001$ and $r_s = 0.558$, $P < 0.001$ respectively), in healthy controls ($r_s = 0.456$, $P < 0.001$ and $r_s = 0.520$, $P < 0.001$ respectively), and in BV positive women ($r_s = 0.698$, $P < 0.001$ and $r_s = 0.687$, $P < 0.001$ respectively).

Women with a high number of neutrophils (⩾75th percentile, average of 13.9 neutrophils on five fields, see Materials and methods) were 15% (9/60) of the healthy group and 37% (19/51) of the BV positive group ($P = 0.007$). Not all women with a high number of neutrophils showed concentrations of proinflammatory cytokines >75th percentile. Table II shows that, among women with high neutrophil counts, the percentage of strong IL-1β responders is much higher in the BV group than in the healthy controls. In addition, a higher percentage of BV positive women had elevated concentrations of both IL-8 and IL-1β.

Concentrations of vaginal IL-8 and IL-1β were strongly correlated in all enrolled women ($r_s = 0.694$, $P < 0.001$), in healthy ($r_s = 0.677$, $P < 0.001$) and in BV positive women ($r_s = 0.735$, $P < 0.001$). However, not all women with high concentrations of IL-8 had high neutrophil counts and/or IL-1β concentrations (Table III). Elevated concentrations of IL-8 were found in 20% (12/60) of healthy and in 31% (16/51) of BV positive women ($P = 0.169$). The percentage of BV positive

Table I. Levels of immune factors in vaginal fluid from healthy and bacterial vaginosis (BV) positive women

Variable	Healthy ($n = 60$)	BV ($n = 51$)	*P* value[a]
No. neutrophils[b]	4.0 (1.2–9.7)	6.3 (1.0–24.7)	0.171
IL-8 (pg/ml)	721 (388–2356)	1584 (289–3585)	0.189
IL-1β (pg/ml)	11 (3–37)	211 (24–624)	< 0.001

[a]Mann–Whitney *U*-test.
[b]Average number of five fields examined at ×1000 magnification of the Gram-stained smear.
Values are median and interquartile range (25th–75th percentile).

Table II. Percentage of healthy and bacterial vaginosis (BV) positive women with innate factors over the 75th percentile among women with neutrophils >75th percentile (average number ⩾13.9)

Variable	Healthy (n = 9) % (n)	BV (n = 19) % (n)	P-value[a]
High IL-8[b]	55.5 (5)	53 (10)	0.885
High IL-1β[c]	0 (0)	84 (16)	<0.001
High IL-8 and IL-1β[d]	0 (0)	42 (8)	0.021

[a]χ^2-test.
[b]IL-8 concentration ⩾2842 pg/ml.
[c]IL-1β concentration ⩾220 pg/ml.
[d]IL-8 concentration ⩾2842 pg/ml and IL-1β concentration ⩾220 pg/ml.

Table III. Percentage of healthy and bacterial vaginosis (BV) positive women with innate factors >75th percentile among women with IL-8 >75th percentile (⩾2842 pg/ml)

Variable	Healthy (n = 12) % (n)	BV (n = 16) % (n)	P-value[a]
High neutrophils[b]	42 (5)	62.5 (10)	0.274
High IL-1β[c]	25 (3)	81 (13)	0.003
High neutrophils and IL-1β[d]	0 (0)	42 (8)	0.004

[a]χ^2-test.
[b]Average count on 5 fields ⩾13.9 pg/ml.
[c]IL-1β concentration ⩾220 pg/ml.
[d]Neutrophils average count on five field ⩾13.9 pg/ml and IL-1β concentration ⩾220 pg/ml.

Table IV. Percentage of healthy and bacterial vaginosis (BV) positive women with innate factors >75th percentile among women with IL-1β >75th percentile (⩾220 pg/ml)

Variable	Healthy (n = 3) % (n)	BV (n = 25) % (n)	P-value[a]
High neutrophils[b]	0 (0)	64 (16)	0.034
High IL-8[c]	100 (3)	52 (13)	0.112
High neutrophils and IL-8[d]	0 (0)	32 (8)	0.246

[a]χ^2-test.
[b]Average count on five fields ⩾13.9 pg/ml.
[c]IL-8 concentration ⩾2482 pg/ml.
[d]Neutrophil average count on five fields ⩾13.9 pg/ml and IL-1β concentration ⩾220 pg/ml.

women with high concentrations of both IL-8 and IL-1β was 3-fold higher than that found in healthy controls (Table III).

High concentrations of IL-1β (⩾220 pg/ml, 75th percentile) were found in 5% (3/60) of healthy and in 49% (25/51) of BV positive women (P < 0.001). Table IV shows that, among women with high values of IL-1β, the percentage of BV positive subjects with a high number of neutrophils is higher than that found in healthy controls.

Table V shows concentrations of innate immunity factors found in subgroups of BV positive women based on low (<75th percentile) and high (⩾75th percentile) values of neutrophils, IL-8 and IL-1β.

Table Va shows the comparison between subgroups of BV positive women with a low (n = 32) and a high (n = 19) number of neutrophils. Considering the median values, women with an elevated number of neutrophils had an ~9-fold increase in IL-8, and an 11-fold increase in IL-1β. All such differences were highly statistically significant.

Table Vb shows the comparison between subgroups of BV positive women with low (n = 35) and high (n = 16) concentrations of IL-8. Women with elevated vaginal IL-8 had a nearly 9-fold increase in number of neutrophils, and near 7-fold increase in concentrations of IL-1β. All such differences were highly statistically significant (P < 0.001).

Table Vc shows the comparison between subgroups of BV positive women with low (n = 26) and high (n = 25) concentrations of IL-1β. Women with high vaginal IL-1β had a 19-fold increase in the number of neutrophils, and an 8-fold increase in IL-8 concentrations. All such differences were highly statistically significant (P < 0.001).

In addition, P values reported in Table V indicate statistically significant differences between each specific subgroup of BV positive women and healthy controls whose values were reported in Table I.

Discussion

The innate host response is the first-line mucosal immune defence and is considered critically important to clear the mucosa of pathogens. Neutrophils are the most important effectors of the innate defence devoted to microorganism clearance. The activated epithelial cells can orchestrate the neutrophil recruitment and activation by secreting chemokines. Innate immunity is also a necessary precursor to an adaptive immune response (Medzhitov and Janeway, 1999). A few

Table V. Levels of immune factors in vaginal fluid of subgroups of bacterial vaginosis (BV) positive women with low (<75th percentile), and high (⩾75th percentile) levels of innate immunity factors

(**a**) Variable	Low neutrophils (n = 32)	P-value[a]	High neutrophils (n = 19)	P-value[a]	P-value[b]
No. neutrophils[c]	1.1 (0.2–6.2)	0.017	26.7 (19.1–31.0)	< 0.001	< 0.001
IL-8[d]	379 (221–1579)	0.192	3373 (2358–8199)	< 0.001	< 0.001
IL-1β[d]	73 (12–313)	< 0.001	773 (296–1958)	< 0.001	< 0.001
(**b**) Variable	Low IL-8 (n = 35)	P-value[a]	High IL-8 (n = 16)	P-value[a]	P-value[b]
No. neutrophils[c]	3.0 (0.3–15.4)	0.492	26.1 (8.8–31.0)	< 0.001	< 0.001
IL-8[d]	480 (243–1666)	0.170	6886 (3760–8371)	< 0.001	< 0.001
IL-1β[d]	105 (15–394)	< 0.001	696 (239–1698)	< 0.001	< 0.001
(**c**) Variable	Low IL-1β (n = 26)	P-value[a]	High IL-1β (n = 25)	P-value[a]	P-value[b]
No. neutrophils[c]	1.0 (0.2–6.4)	0.034	19.1 (7.2–31.0)	< 0.001	< 0.001
IL-8[d] (pg/ml)	368 (186–986)	0.028	2970 (2110–5988)	< 0.001	< 0.001
IL-1β[d] (pg/ml)	28 (8–119)	0.017	624 (374–1604)	< 0.001	< 0.001

[a]Comparison between the BV subgroup and healthy controls by Mann–Whitney U-test.
[b]Comparison between BV subgroups by Mann–Whitney U-test.
[c]Average number of five fields examined at ×1000 magnification of the Gram-stained smear.
Values are median and interquartile range (25th–75th percentile).

studies have measured cytokines in the vaginal fluid and it is not yet established which vaginal marker better indicates the severity of an ongoing vaginal flora perturbation (Donders *et al.*, 2000). No previous study has examined the relationship of vaginal IL-8 with IL-1β, and that of these two proinflammatory cytokines with vaginal neutrophils in BV positive women.

We examined a group of healthy and BV positive non-pregnant women to avoid confounding effects due to pregnancy. We found that, irrespective of BV status, vaginal neutrophils and the proinflammatory cytokines, IL-8 and IL-1β, are highly correlated. It is of note that IL-8 concentrations (median value of 721 pg/ml) are rather high in healthy women, whereas IL-1β concentrations are low (median value of 11 pg/ml). BV status causes a dramatic increase of IL-1β concentrations (~20-fold), showing that the innate immune system is reacting strongly and trying to fight abnormal microbial colonization, although most BV positive women do not show any inflammatory sign. In fact IL-8 and neutrophils are not increased in BV positive women. These findings suggest that the BV microbial consortium produces virulence factors that specifically inhibit IL-8 more than IL-1β. The resulting low IL-8 levels may be responsible for the low counts of vaginal leukocytes and for the clinically observed absence of inflammatory symptoms in most women with BV. However, still unknown bacterial effects could further impair other chemokines and/or directly prevent neutrophil recruitment in BV positive women.

Our data indicate that a finding of high numbers of vaginal neutrophils has different meanings in healthy and in BV positive women. In fact, elevated (⩾75th percentile) numbers of neutrophils in healthy women do not correspond to elevated (⩾75th percentile) IL-1β concentrations (0% of cases), whereas in BV positive women they are nearly coincident (84% of cases) with high IL-1β concentrations. By contrast, high neutrophil counts are not indicative of differences in elevated concentrations of IL-8 in healthy compared to BV positive women (55.5 versus 53% of cases).

Overall, the subgroup of BV positive patients showing high numbers of neutrophils has an ~10-fold increase in proinflammatory cytokine concentrations when compared to the subgroup with low numbers of neutrophils: this implies that some women with BV do experience a strong activation of the innate response.

In our study, the IL-8 median concentration and the number of women with elevated concentrations of IL-8 (⩾75th percentile) were not significantly different between healthy and BV positive women. However, when high concentrations of IL-8 are found in BV positive women, high concentrations of IL-1β (81% of cases) are also present. This suggests that most BV positive women with high IL-8 levels are experiencing an activated innate immunity status, whereas in control women only 25% of the high IL-8 responders have concomitantly high IL-1β concentrations.

In contrast with IL-8, high (⩾75th percentile) vaginal concentrations of IL-1β are a strong indication that a major event altering the vaginal ecology is occurring. In fact only 5% of controls had IL-1β concentrations >75th percentile, whereas 49% of subjects among BV positive women (49%) had elevated IL-1β concentrations. IL-1β is considered a master cytokine of the inflammatory response (Dinarello, 1996) and also a natural adjuvant for adaptive immunity (Staats and Ennis, 1999). This primary inflammatory cytokine induces an array of chemokines, including IL-8, that provides amplification and regulation of innate immunity, with accumulation of leukocytes. The IL-1β rise in BV positive women should activate vaginal innate and adaptive immunity against microbes and is likely to be beneficial to the host as IL-1β should promote an array of cascade responses devoted to fighting the damaging effects of abnormal vaginal colonization.

On the basis of generally accepted notions, high levels of IL-1β should be paralleled by high levels of induced chemokines such as IL-8 (Dinarello, 1996).

Our data strongly suggest that BV-associated factors largely and specifically dampen IL-8, as nearly 50% of BV positive women with elevated IL-1β do not show elevated IL-8. Specific proteases produced by bacteria could be responsible for IL-8 degradation (Cauci *et al.*, 1998). In addition IL-8-producing cells (mainly neutrophils) could be impaired by microbial virulence factors, such as *G. vaginalis* cytolysin (Cauci *et al.*, 1996).

It remains to be established if chemokines other than IL-8, that should be induced by elevated IL-1β concentrations, are also inhibited by BV-associated virulence factors. Conceivably, failure of an increase in chemokine could result in low recruitment and/or activation of neutrophils and impairment of mucosal defences. In

addition, it remains to be established if IL-1β is also partially impaired in some BV positive women, both directly [for example by the action of microbial hydrolytic enzymes (Cauci *et al*., 1998, 2002a)], or indirectly (IL-1β is produced by several types of cells, including neutrophils, so dampening of neutrophil recruitment and/or activation can contribute to reduce IL-1β concentrations).

Some authors have observed that the basal levels of some cytokines are rather high in apparently healthy women (Fidel *et al*., 1997; Eschenbach *et al*., 2000). Different stimuli, including mechanical ones, can trigger neutrophil accumulation and cytokine production (Gallucci and Matzinger, 2001). In the present study, we observed that a finding of elevated levels of vaginal neutrophils or IL-8 in healthy women occurs frequently without a concomitant rise of IL-1β. Conversely, BV positive subjects with high values of neutrophils or IL-8 in vaginal fluid have very high IL-1β concentrations indicating an active mucosal defence. An observation of high neutrophil counts in vaginal fluid has different implications for healthy and BV positive women.

We demonstrated that measurement of IL-1β concentrations in vaginal fluid may discriminate subgroups of BV positive women with impaired versus activated local immune response. It remains to be established if such BV subgroups have different risks of BV-associated complications (Cauci *et al*., 2000, 2002d).

Further studies are required to verify whether other cytokines and chemokines are crucial in rising vaginal immunity against BV-associated microorganisms, particularly those connected with toll-like receptor activation (Akira *et al*., 2001), those that show adjuvant properties for mucosal bacterial antigens (Berzofsky *et al*., 2001), and other proinflammatory cytokines such as TNF-α and IL-6.

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