Lower Levels of Cervicovaginal Tryptophan are Associated with Natural Clearance of Chlamydia in Women.

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ABSTRACT

Chlamydia trachomatis (Ct) infection causes significant morbidity. In vitro studies demonstrate Ct growth inhibition occurs by interferon-gamma (IFN-γ)-mediated depletion of intracellular tryptophan, and some Ct strains utilize extracellular indole to restore tryptophan levels. Whether tryptophan levels are associated with Ct infection in clearance humans remains unknown. We evaluated tryptophan, indole, and IFN-γ levels in cervicovaginal lavages from women with either naturally cleared Ct infection or persisting Ct infection. Women who cleared infection had significantly lower tryptophan levels and trended towards lower IFN-γ levels compared to women with persisting infection. Due to its volatility, indole was not measurable in either group.

KEY WORDS: Tryptophan, Interferon-gamma, Indole, Clearance, Resolution, Chlamydia, Bacterial Vaginosis, Cervicovaginal Lavage

FOOTNOTES

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BACKGROUND

Chlamydia trachomatis (Ct) causes the most prevalent bacterial sexually transmitted infection worldwide [1]. Ct infection can cause chronic inflammation of the reproductive tract, which can lead to complications like tubal factor infertility. Despite public health efforts, Ct infection prevalence continues to increase [1], and our limited understanding of how Ct infection is cleared in humans is a hindrance to vaccine development.

Animal models of chlamydia infection have demonstrated that interferon-gamma (IFN-γ) is essential for infection clearance [2]. Limited human studies support the role of IFN-γ in protective immunity by demonstrating an association between IFN-γ and protection against Ct reinfection [3,4]. In vitro studies demonstrate that IFN-γ induces depletion of intracellular tryptophan [5], which arrests Ct growth (as Ct is a tryptophan auxotroph) and can lead to Ct cell death [6]. To circumvent clearance, Ct has developed survival mechanisms. In response to tryptophan depletion, Ct survives by differentiating into an enlarged, non-infectious form (a process termed "persistence"), which results in a marked decrease in cellular metabolism and cellular division [7]. In addition, urogenital Ct strains encode a functional tryptophan synthase, which can synthesize tryptophan from extracellular precursor substrates, restoring tryptophan levels and permitting Ct survival in the presence of IFN-γ-mediated immune pressure [8]. In vitro studies also demonstrate that indole is a precursor that can be used for tryptophan synthesis [8]. Although unconfirmed in vivo, this relationship may explain why bacterial vaginosis (BV), an infection which may include indole-producing bacteria, is associated with a 2-fold increased risk for incident Ct infection [9]. Whether tryptophan, indole, or IFN-γ levels in the genital tract are associated with Ct infection clearance in humans remains to be elucidated.

To study the relationship between natural clearance of Ct infection and levels of tryptophan, indole, and IFN-γ, we evaluated levels of these compounds in cervicovaginal lavages (CVLs) collected from women with evidence of natural clearance of Ct infection or persisting Ct infection. Natural clearance of Ct infection (*i.e*. resolving infection before treatment) occurs in

approximately 11-44% of women in the interval between a positive Ct screening test and returning for treatment [10]. Although the mechanisms involved in natural clearance are poorly understood, one study reported that women who naturally cleared Ct infection were 4-fold less likely to be re-infected within 6 months, suggesting adaptive immunity (perhaps via IFN-γ-mediated tryptophan depletion) may contribute to natural clearance and protective immunity [11]. The primary objective of our study was to determine whether tryptophan, indole, and IFN-γ concentrations were associated with natural clearance of Ct infection in humans. A secondary objective was to determine whether participant characteristics or concomitant genital infections (e.g., BV) were associated with concentrations of these compounds.

METHODS

Study Population and Procedures. The study population consisted of females ≥16 years of age presenting to the Jefferson County Department of Health (JCDH) Sexually Transmitted Diseases Clinic in Birmingham, Alabama for treatment of a recent positive Ct screening nucleic acid amplification test (NAAT, Aptima Combo 2 [AC2]; Hologic, Marlborough, MA). All patients provided written consent prior to study enrollment. Women who were pregnant, had a prior hysterectomy, were co-infected with HIV, syphilis, or gonorrhea, or had recently received antibiotics with anti-Ct activity were excluded. Participants were interviewed regarding their demographics, symptoms, sexual history, and hormonal contraception use. A pelvic examination was performed to obtain a vaginal swab specimen for wet mount testing to diagnose trichomoniasis, BV (by Amsel's criteria), and candidiasis and an endocervical swab specimen for Ct and *Neisseria gonorrhoeae* testing by AC2 per the manufacturer's instructions. A 10 ml CVL was collected in non-menstruating women by continuously lavaging the cervix, posterior fornix, and vaginal walls with sterile saline for 1 minute using a 3 ml transfer pipette and then was stored at -80°C. All study participants received directly observed therapy with

azithromycin 1 g PO. The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board and JCDH.

Multiplex. IFN-γ was measured in undiluted CVL supernatants using a validated multiplex cytokine assay (V-PLEX, Meso Scale Diagnostics, Rockville, MD) according to the manufacturer's instructions.

High Performance Liquid Chromatography – Mass Spectrometry. CVL cellular debris was removed by centrifugation at 16,100 x g for 10 minutes, followed by protein precipitation with four volumes of ice-cold methanol for 30 minutes, repeat centrifugation, rotor-evaporation of supernatants, and reconstitution of dried residues in 500 µl ddH_2O containing 0.1% formic acid. Reconstituted supernatant (10 µl) was injected into a Jupiter 2.0 x 150mm, 4 µ C18 column (Phenomenex, Torrance, CA) and analytes resolved using a 5-95% linear gradient of acetonitrile in 0.1% formic acid at 400 µl/min and 50°C on a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan). Peaks corresponding to tryptophan and indole were then measured by multiple reaction monitoring (MRM) on a SCIEX 4000 Triple Quadrupole Mass Spectrometer (Concord, Ontario, Canada) in the positive ion mode. Nebulization current was set at 3 with a heater interface temperature of 500°C. Curtain and GS1/GS2 gasses were set at 20, 50, and 5 PSI, respectively. Tryptophan was monitored using the MRM transition of 205.1/188.1 with a collision energy, declustering potential, entrance potential, and exit potential of 15 eV, 60 V, 10 V, and 12 V, respectively. Indole was monitored using the MRM transition of 118/91 with a collision energy, declustering potential, entrance potential, and exit potential of 20 eV, 60 V, 10 V, and 12 V, respectively. 50 msec dwell times were used per transition. The limits of quantification for tryptophan and indole were 0.1 ng/ml and 1 ng/ml, respectively. Tryptophan and indole concentrations were derived by comparison to known metabolite standards.

Statistical Analysis. Analyses were performed with SAS software, version 9.4 (SAS Institute, Cary, NC). Natural clearance of Ct infection was defined by a negative Ct result (by AC2) at the enrollment visit, while persisting infection was defined as having a positive Ct result. Participants defined as natural clearance who were Ct-seronegative by EB-ELISA [12] were excluded, as their initial positive AC2 result may reflect Ct exposure without infection. Otherwise, we tested all women classified as natural clearance and a 1:1 matched group of women with persisting infection (matched by age, race, and co-infections). The Wilcoxon signed-rank test was used to compare tryptophan, indole, and IFN-γ levels between women with naturally cleared vs. persisting Ct infection (matched pair groups). Associations of participant characteristics and co-infections with cleared vs. persisting Ct infection was evaluated with the Pearson chi-square test or multinomial logistic regression, as appropriate. Tryptophan, indole, and IFN-γ levels between Ct infection only vs. Ct infection with a co-infection (independent of Ct clearance status for both) were evaluated with the Mann-Whitney-U test. Additional univariate analyses of covariates (age, number of partners, hormonal contraception use, and prior Ct infection) associated with tryptophan, indole, and IFN-γ levels were performed using Mann-Whitney-U tests, Spearman rank correlation, and Kendall's tau-b correlation, as appropriate. A significance level of $P < .05$ was used for all analyses. Only groups with $n > 5$ were analyzed.

RESULTS

Among 245 women enrolled February 2014 – June 2016, 36 (15%) participants had natural clearance of Ct infection and a CVL available for testing; 36 with persisting infection and a CVL available were matched to them (Table 1). The median interval between screening and enrollment was 10 days (range 3 – 24). The median age was 25 (range 16 – 46) and 94% were African American. 30 (42%) women were co-infected with either BV, trichomoniasis, or

vulvovaginal candidiasis. No significant differences in contraception use, prior history of Ct infection, or interval between screening and enrollment was identified between the two groups.

The median CVL tryptophan and IFN-γ levels were 36.4 ng/ml (range 0 – 815.2) and 7.0 pg/ml (range 0 – 504.2), respectively. Compared to women with persisting Ct infection, women with natural clearance of Ct infection had significantly lower CVL tryptophan levels (median 25 ng/ml vs. 54 ng/ml, *P* = .035, Figure 1A) and a trend towards lower IFN-γ levels (median 2.8 pg/ml vs. 16.1 pg/ml, *P* = .076, Figure 1B). Indole levels were below the limit of detection (<1 ng/ml) for all samples. As a control, we spiked CVL specimens with indole prior to extraction and did not detect indole by HPLC-MS, suggesting indole evaporated during the extraction process.

Stratifying tryptophan levels in Ct-infected women (independent of Ct infection clearance status) by co-infection showed that co-infection with BV was associated with an almost 10-fold lower median CVL tryptophan level compared with Ct mono-infection (median 6.9 ng/ml vs. 67.4 ng/ml, *P* <.001, Figure 1C). No significant difference in tryptophan levels between trichomoniasis or vaginal candidiasis and Ct mono-infection was identified. IFN-γ levels did not significantly differ comparing Ct mono-infection and co-infections (Figure 1D).

We then evaluated the relationship between tryptophan or IFN-γ levels with age, race, hormonal contraception, or number of sex partners in the last 3 months and there were no significant associations found (data not shown). However, there was a trend in women with a history of prior Ct infection having a lower tryptophan level (median 22.8 ng/ml [range 0 – 815] vs. 49.0 ng/ml [0.9 – 806], *P* = .0561, data not shown).

DISCUSSION

Natural clearance of Ct infection occurs slowly (~50% after one year [13]), suggesting Ct employs immune evasion mechanisms. Adaptive immunity likely contributes to this process, evidenced by the association between natural clearance of Ct infection and reduced Ct reinfection rates [11]. IFN-γ is crucial for effective Ct clearance in animal models [2], and likely humans [3,4], by signaling tryptophan depletion, as shown in vitro [6]. To our knowledge, our study is the first to provide in vivo evidence that lower cervicovaginal tryptophan levels, and perhaps lower IFN-γ levels, are associated with Ct clearance. Together, our data suggests that persisting Ct infection is associated with elevated IFN-γ and tryptophan levels, the former likely reflecting the host immune response against CT and latter the organism's effort to combat the IFN-γ-mediated depletion of tryptophan by de novo synthesis of tryptophan by a functional tryptophan synthase. Once Ct infection is cleared, host IFN-γ production declines and the heightened tryptophan synthesis is arrested. Our finding of a trend between a history of a prior Ct infection, a known correlate of protective immunity [14], and 2-fold lower tryptophan levels suggests that heightened IFN-γ production in protected individuals hastens Ct clearance which would correlate with lower tryptophan levels.

An interesting finding in our study was the association between BV co-infection and lower CVL tryptophan levels. Given the well-established association of increased Ct incidence rates in women with BV [9], our finding suggests that BV may use tryptophan-independent mechanisms to promote effective Ct immune evasion (*e.g.* BV biofilms, increasing vaginal pH, etc.).

An unexpected finding was that CVL indole was not detected. This is likely due to evaporation during the extraction process as spiked indole was not recovered. However, discovery-based relative quantitation HPLC-MS pilot studies we performed (data not shown) confirmed the presence of high levels of other indole-containing metabolites in CVL specimens (*e.g.,* indolactic acid, indoleacetyl glutamine, etc.), which have been reported by others [15].

Whether these indole-containing metabolites may be a source of indole or whether indole is present in the cervicovaginal environment in women remains to be elucidated.

Our study has several limitations. First, the majority of subjects were African American, representative of our STD clinic population, and it is unknown if vaginal tryptophan or IFN-γ levels differ between races/ethnicities. Also, our study used Amsel's criteria to diagnose BV, which is less specific than the Nugent score and could have overestimated the frequency of BV infections. Although we excluded women classified as naturally cleared Ct infection who were Ct seronegative (positive AC2 from Ct exposure, but not with established infection) to minimize misclassification of natural clearance, it is still possible misclassification may have occurred.

In summary, we demonstrated that natural clearance of Ct infection is associated with lower cervicovaginal tryptophan levels, and possibly lower levels of IFN-γ. Co-infection with BV, and possibly a prior Ct infection, was also associated with lower tryptophan levels. Further studies are needed to understand the interplay between co-infections and metabolites in the cervicovaginal environment.

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FIGURE LEGENDS

Figure 1. Cervicovaginal lavage tryptophan and IFN-γ concentrations in women with natural clearance of Ct infection vs. persisting Ct infection and also Ct mono-infection vs. co-infections. (A) Women who naturally cleared Ct infection had lower CVL tryptophan levels compared to women with persisting Ct infection (median 25 ng/ml vs. 54 ng/ml, *P* = .035, Fig. 1A) and a trend towards lower IFN-γ levels (median 2.8 pg/ml vs. 16.1 pg/ml, *P* = .076, Fig. 1B). Compared to Ct mono-infection (independent of Ct infection clearance status), significantly lower tryptophan levels were seen in women co-infected with BV

(median 6.9 ng/ml vs. 67.4 ng/ml, *P* <.001, Fig. 1C). IFN-γ levels did not differ by co-infection (Fig. 1D). Only co-infections with *n* >5 were analyzed. The horizontal line denotes the median. The Y-axis is shown as a logarithmic scale.

TABLE 1. Characteristics of Study Participants (*n* Pairs = 36)[a]

Characteristic	Total (*n* =72)	Cleared Ct (*n* = 36)	Persisting Ct (*n* = 36)	*P*-value
Age, median (range)[f]	25 (16 – 46)	25 (16 – 46)	25 (16 – 46)	–
Race, *n* (%)[f]				–
AA	68 (94%)	34 (94%)	34 (94%)	
Caucasian	2 (3%)	1 (3%)	1 (3%)	
Other	2 (3%)	1 (3%)	1 (3%)	
Partners prior 3 Mo, median (range)[d]	1 (1 – 4)	1 (1 – 3)	1 (1 – 4)	0.1399[b]
Contraception, *n* (%)[e]	25 (35%)	9 (25%)	16 (46%)	0.0677[c]
Prior Ct, *n* (%)	40 (56%)	18 (50%)	22 (61%)	0.3428[c]
Co-Infection, *n* (%)[f]				–
None (Ct Only)	42 (58%)	21 (58%)	21 (58%)	
BV	15 (21%)	8 (22%)	7 (19%)	
Trichomoniasis	3 (4%)	1 (3%)	2 (6%)	
Candida	10 (14%)	5 (14%)	5 (14%)	
Visit interval[g], median days (range)	10 (3 – 24)	9 (4 – 21)	11 (3 – 24)	0.1438[b]

Abbreviations: AA, African American; Ct, chlamydia infection; Mo, months; BV, bacterial vaginosis.

[a]Cleared Ct and Persisting Ct pairs were matched on age, race, co-infection.

[b]multinomial logistic regression.

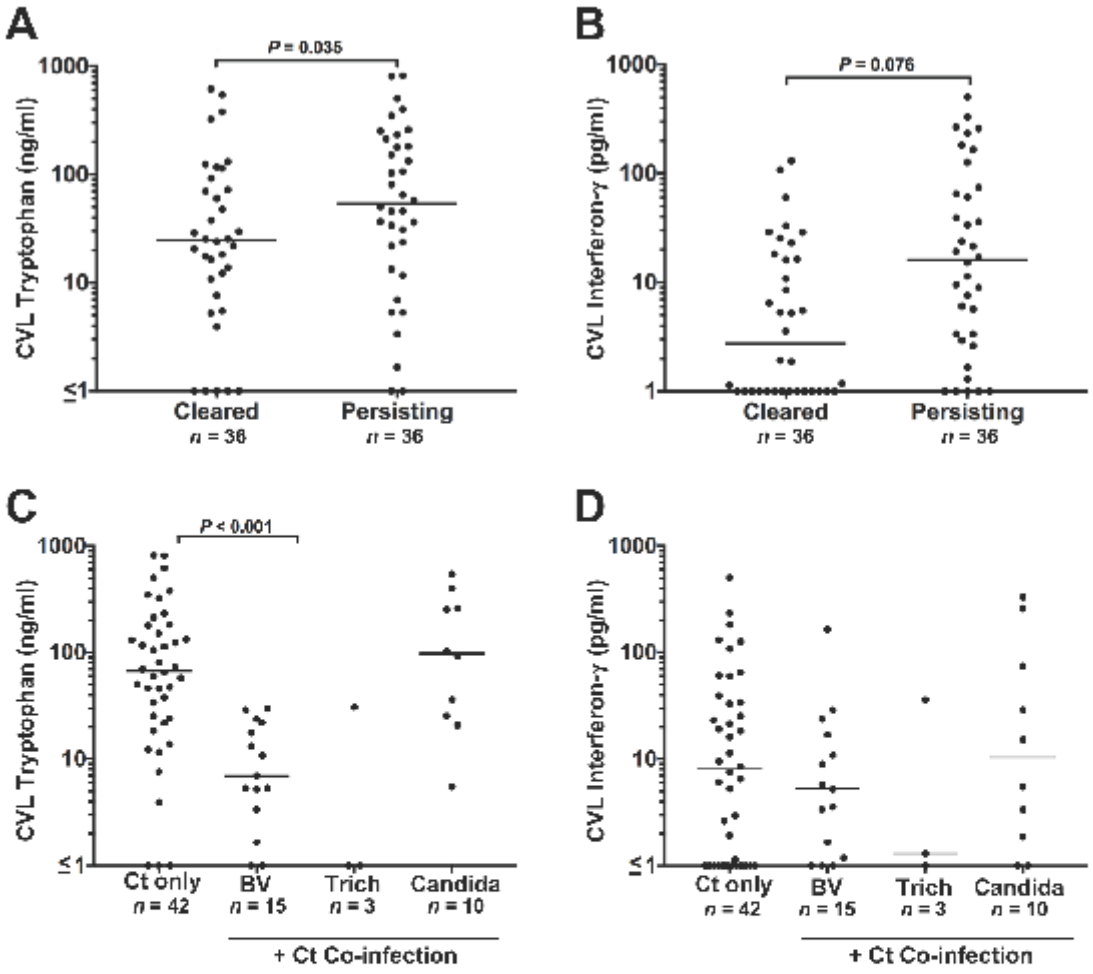
[c]Pearson chi-square test.

[d]Data missing for 3 women.

[e] Data missing for 1 woman.

[f] *P*-value not reported as data was matched.

[g]Duration (days) between screening and treatment visits.


A
P = 0.035
CVL Tryptophan (ng/ml)
1000
100
10
≤1
Cleared
n = 36
Persisting
n = 36
B
P = 0.076
CVL Interferon-γ (pg/ml)
1000
100
10
1
Cleared
n = 36
Persisting
n = 36
C
P < 0.001
CVL Tryptophan (ng/ml)
1000
100
10
≤1
Ct only
n = 42
BV
n = 15
Trich
n = 3
Candida
n = 10
+ Ct Co-infection
D
CVL Interferon-γ (pg/ml)
1000
100
10
≤1
Ct only
n = 42
BV
n = 15
Trich
n = 3
Candida
n = 10
+ Ct Co-infection