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Systemic Cytokine Levels Show Limited Correlation with Risk of HIV-1 Acquisition

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Abstract

It has been hypothesized that immune activation and inflammation may increase HIV-1 susceptibility and that cytokines may be useful biomarkers for risk. Within a prospective cohort, we conducted a nested case-control analysis of plasma cytokine levels among women who acquired HIV-1 <3 months after sampling, compared to three different control groups. We observed associations between lower IL-6 and IL-10 and higher IL-7 levels with HIV-1 acquisition, however these associations were inconsistent when comparing to different control groups. Inconsistent results within our study and among prior studies suggest that reproducible findings are needed before cytokines are useful biomarkers for HIV-1 susceptibility.

Keywords

cytokines; HIV; acquisition; susceptibility; immune activation

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Introduction

Sexual acquisition of HIV-1 is a relatively rare event, and risk varies between individuals as well as over time in the same individual[1,2]. The probability of transmission during any one sex act depends on a complex set of factors in the infected individual, the uninfected individual, and the virus itself. In the exposed individual, susceptibility has been associated with multiple host factors, including immunologic responses and status[3,4], which may vary over time. It has been hypothesized that systemic immune activation and inflammation, known to recruit and activate HIV-susceptible cells, may increase HIV-1 susceptibility. While some studies suggest that increased immune activation increases susceptibility[5,6], others suggest that it may be protective[7,8]. These previous studies compared immune activation markers at a single timepoint in cohorts of high-risk exposed seronegative individuals to uninfected individuals presumed to be HIV-susceptible, without assessing times associated with HIV-1 acquisition. This approach assumes that both the factors measured and HIV-1 susceptibility are static, which is unlikely. Only one study measured immune activation near the time of HIV-1 acquisition - a time of known susceptibility[9]. In that study, immune activation was directly measured in peripheral blood mononuclear cells, and plasma cytokines were also used as a biomarker. The results suggested that women who acquired HIV-1 had higher levels of pro-inflammatory cytokines and activated NK cells than the HIV-exposed seronegative controls, suggesting that suppressing innate immune activation could reduce HIV-1 risk[9].

To further examine relationships between immune activation and HIV-1 acquisition, we conducted a case-control analysis of plasma cytokine levels among women who acquired HIV-1 less than 3 months after sampling, compared to three different control groups: these same individuals at an earlier timepoint when infection did not occur, a random selection of uninfected women, and a group of highly-exposed but uninfected women.

Methods

Study participants

HIV-negative female sex workers in Mombasa, Kenya were enrolled in a prospective cohort[10,11]. Interviews, physical exams and plasma collection occurred monthly before seroconversion. Time of HIV-1 infection was estimated as previously described[10]. Women were included as cases if they had a well-defined HIV-infection date as documented by a pre-seroconversion RNA-positive sample or <30 days between HIV-negative and HIV-positive serology. Case samples, collected between 1993 and 2007, were restricted to <90 days prior to the estimated date of infection (median 24, range 10-90 days). Three control groups were defined. First, external control samples were from women who never seroconverted during follow-up and matched cases on time since enrollment with a 3:1 ratio of controls to cases. Second, a set of control samples, with a similar distribution across calendar year, was chosen from women considered to be relatively resistant to HIV-infection, as they remained HIV-negative during >8 years of follow-up with reported unprotected sex. Third, internal control samples were from case women, but from an earlier timepoint (9-12 months prior to infection).

Ethical approval was obtained from Kenyatta National Hospital in Nairobi, the University of Washington and the Fred Hutchinson Cancer Research Center.

Laboratory Methods

HIV-1 serology was done by ELISA (Detect-HIV; BioChem ImmunoSystems, Montreal) and positive samples confirmed by a second ELISA (either Recombigen; Cambridge Biotech, Worcester, MA or Biorad HIV 1-2; Biorad, Hercules, CA).

Blood was collected in heparinized tubes, plasma was frozen at -80°C and shipped to Seattle. Plasma HIV-1 RNA levels were determined by the Gen-Probe HIV-1 viral load assay (Gen-Probe, San Diego, California)[10].

Cytokine concentrations were determined using Milliplex MAP High Sensitivity Human Cytokine 13-plex (Millipore, Billerica, MA) on Luminex200 (Luminex, Austin, TX). Multiple samples from the same woman were tested on the same plate to avoid inter-assay variability. The lower limit of detection (LOD) for each cytokine was based on a standard curve using a custom export and quality control program in conjunction with Ruminex, a package for use with the R statistics program[12]. Samples with cytokine levels below the LOD were assigned the midpoint between the LOD and zero.

Statistical Analysis

Statistical analysis was performed using Stata9.2 (Stata, Texas, USA). For cytokines in which <80% of the data were above the LOD, data were dichotomized to above or below detection and chi-squared tests were used for cases versus external controls or McNemar's test for cases versus internal controls (matched). For cytokines with >80% of the data above detection, continuous data were analyzed using Mann-Whitney U tests for cases versus external controls or Wilcoxon matched signed-rank tests for cases versus internal controls. A 5% false discovery rate was used to correct for multiple comparisons.

Results

To test the hypothesis that systemic immune activation is correlated with HIV-1 acquisition, we first compared plasma cytokine levels in 162 cases (i.e., women who acquired HIV-1 within 3 months of the sample tested) to 470 external controls (women uninfected throughout follow-up, median 4.8 years, range 0.3-17.8) with year of sample collection (storage time) distributed similarly across groups (Table 1). Overall, these groups appeared similar in terms of known risk factors such as hormonal contraceptive use, frequency of unprotected sex, number of sex partners, and other sexually transmitted infections (STIs) (Table 1). However, compared to the external controls, there were significantly higher proportions of cases with an STI (p=0.005) and depot medroxyprogesterone acetate (DMPA) use (p=0.0013). Neither the presence of an STI, nor DMPA use, were associated with levels of any of the cytokines tested (data not shown). In both the cases and external control groups, plasma cytokine levels were highest for IL-8, IL-10, IL-6 and TNF-α. For all other cytokines, >20% of the data were below the LOD (Table 2). There were no statistically significant differences in levels (for cytokines with >80% of samples detectable)

or % detectable (if <80% of samples were detectable) between cases and external controls (Table 2, columns 3-5).

The external controls described above were randomly chosen from the larger cohort, and some of these external controls were likely susceptible despite remaining uninfected during study follow-up. Therefore, we also compared cytokine levels in women who became infected to women who appeared more resistant to HIV infection. We selected women who remained uninfected despite ≥8 years of follow-up with reported unprotected sex (N=86). The cases and resistant women had similar risk factors at the time of sampling as evidenced by similar sexual risk behaviors, but differed in hormonal contraceptive use by pill ($p<0.001$) or DMPA ($p=0.002$), and in prevalence of STIs ($p=0.049$) (Table 1). Although the average number of unprotected sex acts per week was similar in the two groups, the resistant women remained uninfected for a median 13.4 years of sex work while the cases became infected after a median of 1 year (Table 1). We compared plasma cytokine levels in 162 cases to 86 resistant women (Table 2, columns 3,6). Only IL-7 differed between groups, with a higher proportion of samples with detectable IL-7 in cases versus resistant controls (48% vs 34%, $p=0.03$). As this study was designed to be hypothesis generating, we present unadjusted analysis in Table 2. However, when we controlled for a 5% false discovery rate, the difference was no longer statistically significant.

Finally, we determined whether cytokine levels at the time of HIV-1 acquisition differed from cytokine levels in the same women at a time not associated with HIV-1 infection. Sixty-five of the cases had an additional sample 9-12 months prior to infection, a time unlikely to be associated with infection while minimizing the potential for major changes in other risk factors. Similar to the results described above, most cytokines were detectable in <80% of samples, and the proportion detectable did not differ significantly between cases and their internal controls (Table 2, columns 8,9). Median IL-10 and IL-6 levels were significantly lower at the time of HIV-1 acquisition than in the period 9-12 months prior to infection (Table 2). However, after controlling for multiple comparisons only trends remained ($p=0.13$ and $p=0.12$, respectively).

Discussion

Systemic cytokine levels have been proposed as potential biomarkers for HIV-1 disease progression[13] or acquisition risk[5,9]. However, the reliability of cytokine biomarkers remains unclear as different studies show associations with different cytokines, sometimes in different directions[5–8]. Here we show systemic cytokine levels were not strongly associated with HIV-1 acquisition. In the largest study to date, we compared plasma cytokine levels in women at a time of known susceptibility to three different control groups without consistent results. In women who became HIV-1 infected, we saw evidence of decreased IL-6 and IL-10 levels at a time of known susceptibility compared with an earlier sample. However, these associations were not apparent when comparing women who became infected to women who did not, including a group of women who appeared HIV-1-resistant. When we assessed the association of calendar year of sample collection and cytokine levels, we surprisingly found that increases in length of storage time was associated with an increase in select cytokine levels: IL-7, IL-6 and IL-8 (data not show). Our study was designed to

minimize effects of sample storage time by ensuring that groups of case and control samples had similar calendar year distribution, but we cannot rule out an influence of this on the internal control findings. In addition, it is surprising that lower levels of both IL-6 and IL-10 were associated with acquisition, as these cytokines have opposing functions: IL-6 is a pro-inflammatory cytokine secreted by monocytes and macrophages, while IL-10 is an immunosuppressive cytokine produced by monocytes and regulatory T cells.

We are only aware of one other case-control study which analyzed cytokines at the last pre-infection visit for cases to assess a time of known susceptibility[9]. Despite the facts that our study design was similar, that we used the same high-sensitivity cytokine 13-plex kit, and that our sample size was larger, our results were discordant with this previous study. Naranbahi et al observed higher IL-2, IL-7, IL-12p70 and TNF-α levels in cases compared to controls. Of the associations they observed, we only saw marginal evidence of an association between increased detection of IL-7and HIV-1 acquisition. However, our study did not include analysis of cellular activation which strengthened the results of the Naranbhai study. In addition, our study was limited to 13 cytokines. We cannot rule out that cytokines that are consistently elevated in HIV-infected individuals, such as IP-10 [14–17], may be useful markers for HIV susceptibility.

Other studies showing a correlation between cytokine levels and HIV-1 transmission risk include studies in which intracellular cytokine levels in blood[5] or cytokine levels in genital secretions[6,18] were compared in exposed seronegative individuals versus HIV-negative individuals presumed to be susceptible. There was no overlap in the cytokines associated with infection in these different studies, and samples were not tested in individuals near the time of HIV-1 acquisition. Our data may suggest there are some differences between cytokine levels near acquisition compared to one year prior, which may partially explain differences between studies.

One study that showed an association between genital cytokine levels and protection from HIV-1 in a cohort of highly-exposed seronegative individuals also tested plasma cytokine levels and did not observe a correlation with acquisition[6]. Many studies suggest that systemic cytokine concentrations are not predictive of genital cytokine concentrations[6,16,19], and that local genital inflammation may result in increased HIV-1 susceptible cells at the site of exposure, and thus may better predict transmission. Indeed, genital cytokine levels have been associated with increased HIV-1 shedding[16,19–21], an association that was not reflected at the systemic level[6,16,19]. A limitation of the current study was that genital samples were not available and we may have missed cytokine biomarkers of HIV-1 susceptibility specific to genital secretions.

Another potential limitation is the bead-based cytokine assay used, which we and others have observed shows high inter-assay variability[22–24]. We attempted to minimize this by testing samples from the same woman on the same plate and we intermingled cases and external controls among plates because, in our hands, samples tested on the same plate showed reproducible results.

In this study, our 162 case samples were within a relatively narrow window, 10-90 days, prior to infection. This interval between sample and infection was narrower than the Naranbhai study with a median 127 days (range 15-404) prior to infection. However, it is possible that changes in the immune milieu that affect HIV-1 susceptibility are very transient and could require sample timing be even more refined than was possible here.

In conclusion, we observed evidence of associations between HIV infection and lower concentrations of IL-6, IL-10 and more frequent detection of IL-7. These relationships did not remain statistically significant after controlling for multiple comparisons and were not consistent across different control groups. Our results are not consistent with prior studies, despite our larger sample size and multiple control groups. The inconsistency between studies suggests that plasma cytokine profiles may not yet be useful for tracking HIV-1 susceptibility. In order to identify potential immune correlates for HIV-1 risk, data from larger studies with multiple measures of immune activation in addition to cytokine levels, such as detailed cellular measures from both systemic and genital samples with as tight sampling as possible would be needed. The convergence of markers indicative of the same biological processes within and across studies may provide insight into biological mechanisms underling acquisition and provide support for cytokines as correlates. In the meantime, the utility of cytokines as biomarkers for susceptibility may be limited until a given cytokine is significantly correlated with acquisition across multiple studies.

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Table 1

	Cases	External controls	Resistant women
	n=162	n=470	n=86
Age	28 (24 – 33)	30 (25 – 35)*	33 (29 – 38)*
Years in sex work prior to enrollment	1 (0.25 – 3)	1 (0.1 – 4)	2 (0.5 – 7)
Years in sex work after enrollment (censored at infection)	1.0 (0.4 – 2.2)	4.8 (2.2 – 8.2)*	13.4 (11.2 – 15.6)*
Calendar year of sample collection	1997 (1995 – 2001)	2000 (1996 – 2005)	1997 (1995 – 2002)
Hormonal contraception in past 70 days, no. (%)			
Oral contraceptive pill	28 (17%)	54 (12%)	0 (0%)*
Depot medroxyprogesterone acetate	51 (32%)	92 (13%)*	11 (13%)*
Norplant	3 (2%)	10 (2%)	1 (1%)
Intrauterine device	4 (2.5%)	9 (2%)	2 (4%)
Sexual risk behavior in past week			
Sex frequency	1 (0 – 2)	1 (0 – 2)	1 (0 – 2)
Number sexual partners	1 (0 – 1)	1 (0 – 1)	1 (0 – 1)
Any unprotected sex, no. (%)	43 (28%)	107 (23%)	31 (36%)
Sexual risk behavior averaged over time in cohort			
Average unprotected sex acts per week	0.08 (0 – 0.6)	0.09 (0 – 0.7)	0.1 (0 – 0.7)
STI in past 70 days, no. (%)			
Bacterial vaginosis	85 (52%)	228 (49%)	40 (47%)
Trichomoniasis	13 (8%)	31 (7%)	9 (11%)
Cervicitis	35 (22%)	72 (9%)	12 (14%)
Gonorrhea	16 (10%)	21 (4%)*	2 (2%)
Genital ulcer disease	8 (5%)	10 (2%)	5 (6%)
Any	113 (70%)	271 (58%)*	48 (57%)*

Values shown are median (IQR) except as noted.

*Significantly different than cases, $p<0.05$

Table 2

Plasma cytokine levels compared between cases just prior to HIV-1 infection and HIV-1-uninfected controls

Cytokine	LOD (pg/mL)	Cases n=162	External Controls n=470	p-value [c,d]	Resistant women n=86	p-value [c,e]	Subset of Cases [f] n=65	Internal controls n=65	p-value [c,g]
		% detectable	% detectable		% detectable		% detectable	% detectable	
GMCSF [a]	1.63	26%	24%	0.59	28%	0.74	25%	15%	0.08
IFN-γ [a]	1.67	32%	31%	0.81	29%	0.62	34%	28%	0.41
IL-1β [a]	1.00	14%	18%	0.28	17%	0.50	14%	18%	0.44
IL-2 [a]	1.09	19%	21%	0.60	23%	0.45	17%	18%	0.83
IL-4 [a]	2.06	40%	36%	0.32	31%	0.18	38%	35%	0.69
IL-5 [a]	1.02	16%	14%	0.58	20%	0.46	17%	20%	0.64
IL-7 [a]	1.45	48%	42%	0.15	34%	**0.03**	42%	45%	0.72
IL-12 [a]	2.30	9%	11%	0.48	12%	0.56	9%	15%	0.21
IL-13 [a]	2.30	15%	19%	0.29	14%	0.86	12%	18%	0.16
		Median (IQR)	**Median (IQR)**		**Median (IQR)**		**Median (IQR)**	**Median (IQR)**	
IL-6 [b]	1.64	5.7 (2.7–12.5)	6.0 (2.9–13.4)	0.83	5.8 (3.6, 14.7)	0.51	4.8 (2.8 – 8.3)	8.6 (4.4 – 13.6)	**0.009**
IL-8 [b]	1.02	49.0 (17.5 – 160)	41.9 (14.3–125)	0.19	37.5 (14.6, 105)	0.30	38.1 (20.5 – 98.7)	36.9 (22.8 – 119)	0.88
IL-10 [b]	1.35	13.6 (7.7–26.9)	14.6 (8.7–26.4)	0.37	12.5 (8.1, 23.6)	0.87	10.5 (6.4 – 23.3)	13.8 (7.6 – 31.7)	**0.02**
TNF-α [b]	0.98	6.2 (4.2 – 8.9)	5.85 (4.0–8.71)	0.37	6.4 (4.1, 8.9)	0.89	5.5 (3.5 – 8.2)	6.4 (5.4 – 8.6)	0.07

Abbreviations: LOD, limit of detection; GMCSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; IQR, interquartile range.

[a] Data was dichotomized for cytokines in which <80% of data was above detection

[b] Continuous data was analyzed for cytokines in which >80% of data was above detection

[c] Nominal p-value is reported. Significant associations in bold did not remain significant after controlling for a false discovery rate of 5%.

[d] Difference observed between cases and external controls

[e] Difference observed between cases and resistant women

[f] Subset of cases that had an internal control sample available 9–12 months prior to HIV infection

[g] Difference observed between case subset and internal control samples