

Changes to the cytokine microenvironment in the genital tract mucosa of HIV⁺ women

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SUMMARY

As previous studies have indicated that genital tract mucosal T cell function may be impaired in HIV infection, we investigated the T cell cytokine mRNA in the genital tract mucosa of HIV-infected women to determine if there are alterations in the cytokine profile which may explain the T cell impairment. The *in situ* hybridization technique was used to investigate the T helper-1 (Th1: IL-2, interferon-gamma (IFN- γ)) and Th2 cytokine (IL-4, IL-5, IL-10) mRNA profile in cervical biopsies from 10 HIV⁺ and 10 HIV⁻ subjects. Cervical intraepithelial neoplasia (CIN) and genital infection had previously been excluded and the distribution of immunocompetent cells within the cervical mucosa was known for each subject. Non-parametric tests were used to compare the optical density (OD) of cytokine mRNA in the HIV⁺ and HIV⁻ groups. Comparisons were also made between peripheral CD4 lymphocyte counts, cervical CD4/CD8 T lymphocyte ratios and cytokine mRNA OD in HIV⁺ subjects. The HIV⁺ women had significantly higher mRNA OD for the Th2 cytokines IL-4, IL-5 and IL-10 than HIV⁻ women. There was also significantly lower IL-2 mRNA OD in the former group. HIV⁺ women had lower IFN- γ mRNA than HIV⁻ women, but the difference was not statistically significant. There was no correlation between cytokine mRNA OD and peripheral CD4 count or cervical CD4/CD8 ratio. The predominance of Th2 cytokines, which are immuno-inhibitory, in the cervical mucosa of HIV⁺ women may underlie the impaired cytotoxic potential observed in the CD8⁺ T lymphocytes and may contribute to the susceptibility of HIV-infected women to recurrent genital tract infections and cervical neoplasia.

Keywords HIV female immunology cytokines uterine cervix

INTRODUCTION

The existence of an immune system in the female lower genital tract has been acknowledged for several years [1–3], but its role in the prevention of infection and protection against cervical neoplasia is poorly understood. The rising prevalence of HIV infection in women [4] and the risks of transmission to the offspring [5] have recently focused the attention of researchers on the mechanisms of mucosal transmission of HIV. Epidemiological studies have been successful in identifying some of the factors which predispose to the horizontal [6] and vertical [7,8] transmission of HIV, but there is now a recognition that an understanding of local immune function may be the key to elucidating the mechanism of HIV transmission.

Work undertaken in this Department [9] has shown that HIV infection is associated with alterations in the distribution of

immunocompetent cells in the lower genital tract. In particular, there is an increase in absolute cytotoxic (CD8⁺) lymphocyte proportions in the cervical mucosa when compared with HIV⁻ women. These CD8⁺ cells, although primed (CD45RO⁺), appeared to be functionally impaired, in that they showed little or no expression of cytolytic granules (perforin^{low}, TIA-1⁻). These changes occurred before there was evidence of systemic immune dysfunction. It was postulated that systemic HIV infection may occur because of the inability of the inept T cells to overcome sexually acquired HIV at a local level. It was also suggested that the impaired cytotoxic potential of these cells may underlie the susceptibility of HIV-infected women to recurrent viral and fungal infections of the genital tract and to cervical intraepithelial neoplasia (CIN).

It is, as yet, unclear why these changes in T cell subset distribution occur. It has been shown that HIV infection is associated with changes in the pattern of cytokine production in the peripheral blood [10,11]. However, there have been few attempts to investigate cytokine production in the lower genital

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tract in HIV-infected females. Belec *et al.* [12] showed that levels of proinflammatory cytokines were raised in cervicovaginal secretions of HIV-infected women. It may be that changes also occur in T cell cytokine production in the genital tract which may underlie the alterations in immunocompetent cell distribution observed in the cervical mucosa of HIV⁺ women.

Two distinct patterns of cytokine were originally defined among a panel of mouse T cell clones [13]. T helper-1 (Th1) cells produce IL-2 and interferon-gamma (IFN- γ), while Th2 cells express IL-4, IL-5 and IL-10. Evidence from strong immune responses in both mice and humans suggests that Th1 and Th2 cell patterns both occur and are important *in vivo* [14,15]. Th1 responses are usually immunostimulatory, while Th2 responses are inhibitory. The response pattern induced by a particular pathogen is usually predictable and appropriate [16], but there are severe consequences if an incorrect pattern is induced. HIV establishes a close interaction with the immune system that can result in increased or decreased production of various cytokines [10]. It has been suggested that T lymphocytes in the peripheral blood of HIV-infected individuals switch from producing Th1 cytokines to producing Th2 cytokines. This switch from a Th1 to Th2 cytokine production pattern is said to favour progression to AIDS [17,18]. Altered patterns of cytokine production have been described at other mucosal surfaces in HIV infection [19], but there are few studies investigating cytokine production in the lower genital tract in HIV-infected women. We therefore decided, using the technique of *in situ* hybridization, to study the Th1, Th2 cytokine mRNA profile in cervical biopsies from HIV-infected women and to compare these with cervical biopsies from HIV⁻ women, with a view to elucidating further the immune mechanisms of the lower genital tract and changes associated with HIV infection.

SUBJECTS AND METHODS

Subjects

A group of 10 HIV⁺ women at varying stages of disease and a group of 10 HIV⁻ volunteers with documented negative HIV antibody tests were recruited to the study. The HIV⁺ women were out-patient attenders of the Ian Charleson Centre, the dedicated HIV unit of the Royal Free Hospital. The HIV⁻ women were recruited from the same-day HIV antibody testing clinic, also based at the Ian Charleson Day Centre. The study had received prior approval from the Hospital Ethics Committee and informed consent was obtained in all cases. Demographic details including social, sexual and menstrual history were available for all subjects, and genital infection and CIN had previously been excluded. As previously described [9], a biopsy of the squamo-columnar junction of the cervix was obtained from each subject during the proliferative phase of the menstrual cycle. Peripheral blood was collected by venepuncture from all HIV⁺ subjects on the same day as the cervical biopsy was taken.

Preparation of biopsy specimens

The biopsy specimens were frozen in liquid nitrogen, cut and fixed as previously described [9]. The cut sections, mounted on poly-L-lysine coated slides, were wrapped back to back in cling film and stored at -20°C until required. Prior staining of sections from each biopsy with haematoxylin and eosin established that there was no histological abnormality in biopsies from both groups. The distribution of immunocompetent cells, including the CD4/CD8 T cell

ratios, had been similarly established for biopsy sections from each group [9].

In situ hybridization

mRNA for IL-2, IFN- γ , IL-4, IL-5 and IL-10 were studied. Tissue fixation was performed by immersing the slides in 4% paraformaldehyde for 10 min. The sections were then pretreated by immersing them in 40 ml 10 \times PBS + 2 ml 1 M MgCl₂ + 358 ml DEPC-H₂O (PBS-M) for 10 min and in PBS-M + 0.25% Triton X-100 + 0.25% Nonidet P40 twice for 5 min each time. Acetylation of the specimens was performed by dipping the slides in 20% acetic acid at 4°C for 15 s. The sections were then incubated in 20% glycerol for 1 h. Hybridization was performed by placing the slides in a moist chamber and covering each section with 30 μ l of the probe at an appropriate dilution in prehybridization solution. They were incubated for 20 min at 70°C for 20 min and at 37°C overnight. In order to demonstrate that the target nucleic acid was RNA, controls were prepared in which biopsy sections were pretreated with RNase before application of the specific oligonucleotide probe. Additionally, positive controls in which sections were incubated with poly-dT oligonucleotide probes which detects all animal mRNAs were prepared. Simultaneous negative controls in which the specific oligonucleotide probe was omitted were also used.

The slides were washed after hybridization to remove non-specifically bound RNA before detection. Digoxigenin (DIG)-labelled synthetic single-stranded DNA oligonucleotide probes (R&D Systems, Abingdon, UK) were used to identify mRNA for the cytokines of interest and detection was carried out with anti-DIG antibody (Boehringer, East Sussex, UK). The slides were developed with 5-bromo-4-choloro-3-indolyl phosphate (BCIP)/NBT (Sigma, Poole, UK). After developing, the slides were washed in de-ionized water to remove the coverslip. They were wiped carefully and mounted in PBS glycerol.

mRNA expression was quantified on each section by measurement of optical density (OD) using a monochrome video camera attached to a microscope with a \times 40 objective. The relative density of the reaction product was recorded using a computerized image analyser (Seescan, Cambridge, UK). Frames were drawn around positively staining cells as previously described [9], selecting three areas per slide. The software automatically calculates the area within the frame and the OD per unit area is generated by the software. The OD of all positively staining cells within each frame was measured and the average OD per biopsy section calculated. Each section was independently analysed by two observers (A.O., L.W.P.). Comparisons were made between the HIV⁺ and the HIV⁻ group for each cytokine studied.

Statistical analysis

Unless otherwise stated, the median values, with the range in parentheses, are given. The Mann-Whitney *U*-test was used to compare values from the HIV⁺ and HIV⁻ samples. A probability value (*P*) of ≤ 0.05 was taken as indicating statistical significance. Spearman rank correlation was used to assess the correlation between cytokine mRNA expression in cervical biopsies and other immune parameters in the cervix and peripheral blood. All tests of significance were two-sided.

RESULTS

Demography of study population

The HIV⁺ and HIV⁻ women were similar in terms of age, sexual

Table 1. Demographic and lifestyle details of HIV⁺ and HIV⁻ subjects

Demographic details	HIV ⁺ , n = 10	HIV ⁻ , n = 10
Median age in years (range)	31 (22–47)	31.5 (26–41)
Median age at menarche (range)	13.5 (11–16)	12 (12–16)
Median age at coitarche (range)	19 (14–21)	15.5 (14–21)
Mean number of sexual partners (s.d.)	6.0 (4.9)	10.4 (6.5)
Previous STD (%)		
None	6 (60.0)	8 (80.0)
Warts	1 (10.0)	1 (10.0)
Herpes	3 (30.0)	1 (10.0)

and menstrual history (Table 1). All the HIV⁺ women acquired HIV through heterosexual intercourse. Table 2 shows the disease stage in the HIV⁺ women. All HIV⁺ women used condoms for contraception, but two women used the combined oral contraceptive pill (cocp) in addition. Two of the HIV⁻ women used the cocp, while the others relied on condoms. One woman from each group smoked cigarettes, each smoking over 20 cigarettes a day.

Cytokine mRNA expression

The median OD measurements for each of the cytokines under study are shown in Table 3. mRNA for the Th1 cytokines IL-2 and IFN- γ and the Th2 cytokines IL-4, IL-5 and IL-10 was present in biopsy sections from both the HIV⁺ and HIV⁻ groups. However, there was significantly less mRNA for IL-2 detected in cervical biopsies from the HIV⁺ subjects compared with the HIV⁻ subjects. Although there was less IFN- γ mRNA in biopsies from the HIV⁺ group compared with the HIV⁻ group, this difference was not statistically significant. However, there was significantly more mRNA for the Th2 cytokines, IL-4, IL-5 and IL-10 in the cervical biopsies from HIV⁺ compared with HIV⁻ subjects.

Correlates with peripheral blood

No relationship was detected between peripheral CD4 lymphocyte count and cervical cytokine mRNA OD in HIV⁺ women. The number of women who smoked and the number of women who used the combined oral contraceptive in each group was too small for subgroup analysis, assessing the effects of these factors, to be performed.

Table 3. Optical density of cytokine mRNA in cervical biopsies

Cytokine	HIV ⁺	HIV ⁻	P*
	Median (range)	Median (range)	
hIL-2	0.12 (0.09–0.17)	0.20 (0.12–0.30)	0.04
hIL-4	0.28 (0.19–0.54)	0.09 (0.07–0.16)	0.0006
hIFN- γ	0.18 (0.14–0.36)	0.26 (0.15–0.32)	0.50
hIL-5	0.34 (0.28–0.63)	0 (0–0.4)	0.045
hIL-10	0.34 (0.25–0.40)	0 (0–0.39)	0.02

* Mann–Whitney *U*-test.

Correlates with cervical immune parameters

Table 4 shows the peripheral CD4 lymphocyte counts, the cervical CD4/CD8 ratios and the OD for the mRNA of the cytokines under study. As previously demonstrated [9], no relationship was detected between peripheral CD4 counts and cervical CD4/CD8 ratios. In addition, there was no apparent relationship between the cervical CD4/CD8 ratios and the mRNA OD for any of the cytokines under study. The correlation coefficients for cervical CD4/CD8 ratios and cytokine mRNA OD are stated in Table 4.

DISCUSSION

This study demonstrates that there is a predominance of mRNA for Th2 cytokines in the cervix of HIV⁺ women. This observation reflects the Th1 to Th2 shift described in the literature as being associated with HIV disease and which is said to predispose to HIV disease progression [20].

Th1 and Th2 responses occur naturally, and may be an immunologically appropriate response to invasion by certain pathogens. For instance, there is experimental evidence to show sequential production of Th1 and Th2 cytokines in response to live bacille Calmette–Guérin (BCG) [14]. The initial response was dominated by macrophage cytokines (monokines), followed by a marked production of Th1 cytokines (IL-2, IFN- γ) at days 4–5. Later in the reaction (days 10–12) a Th2 response, with IL-4, IL-5 and IL-10 production, was observed. There is substantial evidence of cross-regulation between the Th1 and Th2 Th cell subsets [16]. For example, IL-10 inhibits synthesis of cytokines by Th1 cells which can result in decreased proliferation. IFN- γ , which is itself inhibited by IL-10, inhibits the growth of Th2 cells and IL-4

Table 2. Disease stage of HIV⁺ subjects

Subject no.	Disease stage	Peripheral CD4 count ($\times 10^6/l$)	CD4/CD8	Medication
1	AIDS	210	0.09	AZT, Rifampicin, Co-trimoxazole
2	Asymptomatic	880	0.75	None
3	Symptomatic	330	0.3	Co-trimoxazole
4	Asymptomatic	760	0.6	None
5	Asymptomatic	400	0.34	Didanosine, AZT, acyclovir
6	AIDS	30	0.03	Co-trimoxazole, Prednisolone
7	AIDS	20	0.086	Co-trimoxazole, AZT, Ganciclovir, acyclovir
8	Symptomatic	550	0.6	Acyclovir
9	AIDS	10	0.01	Co-trimoxazole
10	Symptomatic	120	0.11	Co-trimoxazole

Table 4. Relationship between cervical cytokine profile, CD4/CD8 ratios in cervix and peripheral CD4 counts of HIV⁺ subjects

Peripheral CD4 × 10 ⁶ /ml	Cervix					
	CD4/CD8	IL-2	IL-4	IFN- γ	IL-5	IL-10
880	0.25	0.11	0.48	0.14	0.33	0.25
760	0.29	0.17	0.19	0.15	0.35	0.40
550	0.59	0.09	0.54	0.20	0.33	0.35
400	0.17	0.17	0.28	0.34	0.554	0.33
330	0.15	0.12	0.21	0.14	0.28	0.35
210	0.39	0.09	0.28	0.31	0.63	0.34
120	0.41	0.12	0.23	0.20	0.32	0.36
30	0.29	0.09	0.2	0.36	0.32	0.30
20	0.23	0.15	0.30	0.20	0.35	0.33
10	0.20	0.14	0.35	0.14	0.37	0.35
Correlation coefficient cervical CD4/CD8 and mRNA (<i>r</i>)		0.012	0.16	0.12	0.006	0.08

preferentially stimulates the growth of Th2 cells. Sander *et al.* [14] therefore concluded that the production of Th2 cytokines late in the response to BCG may reflect a dynamic *in vivo* balance, preventing over-expression of inflammatory, potentially tissue-damaging Th1 cytokines.

However, HIV infection is chronic and the presence of infectious virus has been demonstrated in female genital tract secretions at all stages of disease [21–23]. The observation of a predominance of Th2 cytokines may therefore not necessarily reflect an HIV-induced pathological shift in cytokine production, but may be an exaggeration of a normal process. The chronicity of HIV, with the constant background antigenic stimulation, may lead to an accumulation of virally stimulated cells which produce Th2 cytokines. This theory is consistent with the observation in a previous publication that there is an accumulation of 'primed' lymphocytes in the cervix of HIV⁺ women [9]. Paradoxically, the accumulation of Th2 cytokines may predispose to the chronicity of the viral infection. IL-10 inhibits Th1 cytokine synthesis, and as murine cytotoxic T lymphocytes synthesize the Th1 pattern of lymphocytes [24], and the same may occur in humans, IL-10 may have a suppressive effect on their ability to kill the virus. Other conditions, such as IgA nephropathy, have been described where an excess of Th2 cytokine production is associated with a disease process [15,25]. No correlation was observed between cytokine levels and HIV disease stage as defined by peripheral CD4 lymphocyte counts, but there may be other confounding and regulating factors (such as the use of anti-retroviral therapy) which were not directly studied. In addition, the small subject numbers mean that non-linear relationships may not be apparent.

Finally, the susceptibility of HIV⁺ women to cervical neoplasia and recurrent genital tract infections may, in part, be explained by this cytokine pattern. Th1 cytokines are immunostimulatory and are thus capable of limiting tumour growth and arresting viral and fungal infection, while Th2 cytokines are immuno-inhibitory and are capable of stimulating tumour growth [26]. Thus the predominance of Th2 cytokines in the cervical tissues of HIV⁺ women may permit neoplastic change, either alone or in conjunction with other cofactors such as human papillomavirus (HPV). Clerici *et al.* [26] demonstrated that peripheral blood mononuclear cells (PBMC) from women with extensive CIN showed reduced production of

IL-2 when stimulated by mitogens when compared with PMBC from women with localized CIN or healthy controls. PMBC from the group with extensive disease showed an increased production of IL-4 and IL-10 compared with the healthy group or those with localized disease. These findings lend weight to the theory that a predominance of Th2 cytokines, either locally or in the peripheral circulation, permits neoplastic change and predisposes women to CIN.

The caveat must be made, however, that the observation of increased mRNA for a particular cytokine may not necessarily be reflected in increased production of that cytokine. It is important, therefore, to study cytokine production in the cervix of HIV⁺ women and to relate this to the cytokine mRNA. In addition, it is not clear whether the mRNA cytokine changes observed are HIV-induced or if some other cofactor, such as HPV, is involved. Studies have shown that the prevalence of HPV16 and 18, which are considered to be of high oncogenic potential, is increased in the cervix of HIV⁺ women with or without CIN [27,28]. Further studies, involving larger subject numbers, relating the presence of HPV to local immune dysfunction may help to elucidate further the mechanisms of genital tract immune function.

In conclusion, therefore, the observation of an altered cytokine profile in the cervix of HIV-infected women is consistent with the previously reported finding of functionally impaired, virally stimulated cytotoxic T lymphocytes within the cervical mucosa. The observed alterations in cytokine profile and cytotoxic T cell phenotype may explain why HIV-infected women are susceptible to recurrent viral and fungal infection of the genital tract and CIN.

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