Gonococcal Cervicitis Is Associated with Reduced Systemic CD8+ T Cell Responses in Human Immunodeficiency Virus Type 1–Infected and Exposed, Uninfected Sex Workers

Rupert Kaul,1,2,a Sarah L. Rowland-Jones,2 Geraldine Gillespie,2 Joshua Kimani,1 Tao Dong,2 Peter Kiama,1 J. Neil Simonsen,3 Job J. Bwayo,1 Andrew J. McMichael,2 and Francis A. Plummer1,3

1Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya; 2Medical Research Council Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom; 3Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada

Neisseria gonorrhoeae cervicitis and human immunodeficiency virus (HIV) type 1 frequently coinfect core transmitter populations, such as female sex workers. Gonococcal cervicitis is associated with increased viral shedding and plasma viremia in HIV-1–infected women and increased HIV-1 susceptibility in uninfected women. We studied the influence of gonococcal cervicitis on CD8+ interferon (IFN)–γ responses to HIV-1 and cytomegalovirus (CMV) epitopes in HIV-1–infected and in highly-exposed, persistently seronegative (HEPS) female sex workers. In HIV-1–infected women, gonococcal cervicitis was associated with reduced IFN-γ responses in bulk CD8+ lymphocyte populations, and intracellular cytokine staining, combined with class I major histocompatibility complex (MHC)–peptide tetramer studies, demonstrated reduced IFN-γ production by HIV-1 epitope–specific CD8+ lymphocytes. In HEPS sex workers, cervicitis was associated with the transient loss of systemic HIV-1–specific CD8+ responses and with reduced function of CMV-specific CD8+ lymphocytes. Impaired function of virus-specific CD8+ lymphocytes may partly explain the deleterious effects of gonococcal cervicitis on HIV-1 immune control and susceptibility.

Despite ongoing education and prevention efforts, the AIDS pandemic continues to progress in sub-Saharan Africa. Sexually transmitted diseases (STDs) frequently coexist with human immunodeficiency virus (HIV) type 1 infection in core transmitter groups, such as female sex workers, and are important in virus transmission dynamics [1, 2]. In particular, cervicitis due to Neisseria gonorrhoeae is common among sex workers [1]. Gonococcal cervicitis in HIV-1–infected women is associated with reversible increases in viral shedding from the genital tract [3, 4] and plasma virus load [5, 6]. These observations suggest that gonorrhea, a mucosal bacterial infection, may adversely affect systemic immune control of HIV-1.

Although the increased plasma viremia seen during gonococcal cervicitis may relate to increased activation of circulating CD4+ lymphocytes [5], cervicitis has also been associated with increased plasma levels of interleukin (IL)–4 and IL-10 [6]. These Th2 cytokines negatively regulate the Th1 cytokines that are important in the generation and maintenance of cellular immunity, including cytototoxic T lymphocyte (CTL) responses [7]. HIV-1–specific CTL play a key role in the control of HIV-1 vir- emia [8], and they have also been detected in the blood and genital tract of highly-exposed, persistently seronegative (HEPS) female sex workers, where the waning of CTL has been associated with late HIV-1 seroconversion [9]. This suggests that HIV-1–specific CTL are important in the protection from both HIV-1 disease progression and HIV-1 acquisition.

We hypothesized that the deleterious effects of N. gonorrhoeae cervicitis on HIV-1 virus load might relate to a fall in systemic HIV-1–specific CTL frequency and/or function. Furthermore, we hypothesized that cervicitis in HEPS women might lead to a reduction in the frequency and/or function of protective HIV-1–specific CTL, thereby enhancing HIV-1 susceptibility. To test these hypotheses, we examined the relation-
ship between *N. gonorrhoeae* cervicitis and systemic HIV-1–specific CD8+ lymphocyte responses in a well-defined cohort of Kenyan sex workers.

**Subjects and Methods**

*Study populations.* Women were enrolled through a dedicated sex worker clinic in the Pumwani district of Nairobi, Kenya [10]. All HIV-1–seronegative sex workers were confirmed to be HIV-1 uninfected by means of a polymerase chain reaction (PCR) system that uses primers for *env, nef,* and *vig* HIV-1 provirus genes specifically adapted to detect African clades [11]. Sex workers were classified as HIV-1 resistant if they were seronegative at enrollment and remained seronegative and PCR negative for HIV-1 infection for >3 years while continuing as a sex worker [10].

*Experimental methods.* CD8+ interferon (IFN)-γ responses to predefined HIV-1 and cytomegalovirus (CMV) peptide epitopes were studied in cryopreserved peripheral blood mononuclear cells (PBMC), using the enzyme-linked immunospot (ELISPOT) assay [12, 13] and intracellular cytokine staining after in vitro peptide stimulation. The intracellular cytokine staining was combined with class I major histocompatibility complex (MHC)–peptide tetramer staining when appropriate tetramers were available [14]. Epitope selection was based on responses in an earlier cross-sectional study [12]. The predefined HIV epitopes used, and their MHC restriction, included SLYNTVATL (A2 p17), KAFSPEVIPMF (B57/B58 p24), LSPRTLNAW (B57 p24), TSTLQEQIW (B57 p24), ILKDPVHGV (B18 Nef), DFRWFKTLRA (B14 p24), ERYLDRQQL (B14 gp41), DLNMMLNIV (B14 p24), and DTVELEDINL (A*6802 Pol). The CMV epitopes included NLVPMVATV (A2 pp65) and TPRVTGGGAM (B7 pp65). Because of limitations in cell numbers, not all assays were performed at all time points. Where possible, cryopreserved PBMC were used from (1) the patients’ annual resurvey visit prior to gonococcal infection, (2) when the patients reported to the clinic with symptoms suggestive of gonococcal infection, and (3) at the patients’ next clinic visit after appropriate therapy. *N. gonorrhoeae* infection was confirmed in all cases by bacterial culture on Thayer-Martin medium. Women with either a positive cervical PCR for *N. gonorrhoeae* or genital ulcer disease were excluded.

In the modified ELISPOT assay [12, 13], 96-well nitrocellulose plates were precoated with IFN-γ–producing antibody 1-DIK (MBTETH), and PBMC were added to duplicate wells, with predefined epitopes at 20 μM, with media alone, or with 1:100 phytohemagglutinin (PHA; Murex Biotech). Plates were incubated overnight at 37°C in 5% CO₂, cells were discarded, and the plate was incubated with biotinylated anti–IFN-γ monoclonal antibody 7-B6-1 biotin (MBTETH), followed by streptavidin-conjugated alkaline phosphatase (MBTETH). IFN-γ–producing cells were detected using an alkaline phosphatase–conjugate substrate kit (BioRad) and were counted using an automated ELISPOT reader (Autoimmune Diagnostika). HIV-1–specific IFN-γ responses were reported as number of spot-forming units (sfu) per 10⁶ PBMC, after correction for background IFN-γ secretion.

Intracellular cytokine assays were performed after epitope stimulation of thawed PBMC, and all samples from each study subject were run and analyzed simultaneously. When available, 0.5–1.5 μL of phycoerythrin (PE)–conjugated class I MHC-peptide tetramers was used to prestain 10⁶ thawed PBMC for 15 min at 37°C, as described elsewhere [14]. Cells were then washed and incubated at 37°C in 5% CO₂, with media alone, with the relevant epitope peptide at 10μM, or with a control peptide. After 45 min, brefeldin A (Sigma-Aldrich) was added to a final concentration of 10 μg/mL, and cells were incubated for a total of 6 h. The cells were made permeable by use of FACS permeabilizing solution (Becton Dickinson Immunocytometry Systems), according to the manufacturer’s instructions, and were stained with monoclonal antibodies IFN-γ–fluorescein isothiocyanate, CD8 peridinin chlorophyll protein, and CD3 allophycocyanin (Becton Dickinson). Stained cells were fixed overnight and analyzed the next day.

For each study subject, the mean CD8+ response frequency when the subject tested negative for gonorrhea (hereafter “gonorrhea-negative visit”) was taken as the baseline and was compared with the mean CD8+ response frequency when the subject tested positive for gonorrhea (hereafter “gonorrhea-positive visit”), using the paired-samples *t* test.

**Results**

Nine HIV-1–infected and 6 HEPS women were enrolled in the study between 1998 and 2000. When possible, assays were done at the subject’s annual follow-up visit prior to gonococcal infection (11 subjects; total of 11 visits), at the time of culture-confirmed cervical infection by *N. gonorrhoeae* (all 15 subjects; total of 18 visits), and at the clinic visit following appropriate therapy (13 subjects; total of 13 visits). Posttreatment samples were obtained a median of 154 days after therapy (range, 23–418 days). Samples were available for 1.2 gonorrhea-positive visits per subject (range, 1–2 visits) and 1.8 gonorrhea-negative visits per subject (range, 1–3 visits). No significant effect of *N. gonorrhoeae* infection on CD4+ or CD8+ counts was seen in HIV-1–infected or HEPS subjects (data not shown).

In HIV-1–infected sex workers, epitope-specific IFN-γ production was lower at gonorrhea-positive visits when assayed using either intracellular cytokine staining (mean, 59% of gonorrhea-negative baseline; *P* = .01; figures 1A and 2A) or the ELISPOT assay (mean, 68% of gonorrhea-negative baseline; *P* = .07). However, the total number of epitope-specific CD8+ lymphocytes, as measured phenotypically using class I MHC-peptide tetramers, did not differ at gonorrhea-positive visits (mean, 96% of gonorrhea-negative baseline; *P* = .8; figure 1B). This suggested that the reduced IFN-γ responses seen using intracellular cytokine staining might be due to functional defects within the population of virus-specific CD8+ lymphocytes.

To examine the effect of gonococcal cervicitis on the function of epitope-specific CD8+ lymphocytes more precisely, we used class I MHC-peptide tetramers to prelabel epitope-specific CTL and then examined IFN-γ secretion by this population after stimulation by the cognate epitope (figures 1B and 2B). IFN-γ secretion by virus-specific CD8+ lymphocytes was significantly reduced at gonorrhea-positive visits (mean, 75% of gonorrhea-
HI-V-1–specific responses are relatively weak in this population [12], generally falling below the level of detection of class I MHC-peptide tetramers or intracellular cytokine staining [13], which precludes the use of these techniques to study CTL function. We therefore used the ELISPOT assay to study HIV-1–specific responses in 4 of these women and tetramers combined with intracellular cytokine staining to study the high-frequency CMV-specific responses detected in the remaining 2 women.

HIV-1–specific responses present at baseline fell below the level of ELISPOT detection at gonorrhea-positive visits in all 4 HEPS women studied (mean CD8+ response frequency, 75 vs. 3 sfu/10⁶ PBMC; P = .04). When the function of epitope-specific CD8+ populations was studied, using tetramers, in the 2 remaining HEPS sex workers, the overall frequency of CMV-specific tetramer responses did not change in relation to gonorrhea status (P = .6; figure 1B). However, there was a strong trend toward reduced IFN-γ secretion both in the overall CD8+ population (mean, 56% of gonorrhea-negative baseline; P = .08; figure 1A) and in the gated population of epitope-specific CD8+ lymphocytes (mean, 71% of gonorrhea-negative baseline; P = .08; figure 1B). Among HEPS sex workers, reported changes in sexual practices (condom use and types of sex or number of partners) were not related to gonorrhea status.

Discussion

In this study, N. gonorrhoeae cervicitis in Kenyan sex workers was associated with reduced systemic CD8+ IFN-γ responses to predefined HIV-1 and CMV epitopes. Reduced CD8+ responses were initially demonstrated using the ELISPOT assay and intracellular cytokine staining, techniques in which the experimental output is a proportion of PBMC or CD8+ lymphocytes that produce IFN-γ in response to a given epitope. This means that expansion of new CD8+ T cell subsets in response to gonococcal infection could result in a relative reduction in CD8+ responses to other epitopes, even if the absolute number of epitope-specific CD8+ T cells remained unchanged. However, this is unlikely to explain our findings for several reasons. First, gonococcal cervicitis was not associated with significant changes in overall peripheral CD4+ or CD8+ lymphocyte counts or with changes in epitope-specific CD8+ T cell population frequencies measured with epitope-specific tetramers. More importantly, functional studies that combined intracellular cytokine staining with class I MHC-peptide tetramers, thereby restricting the analysis to epitope-specific CD8+ lymphocytes, confirmed that gonococcal cervicitis was associated with reduced IFN-γ responses within this population.

Previous study of this sex worker cohort has shown that gonococcal cervicitis, despite being apparently confined to a mucosal site (the genital tract), was associated with increased HIV-1 plasma viremia and increased plasma levels of type 2 cytokines (IL-4, IL-6, and IL-10) [6]. The current study shows that gonococcal cervicitis may also be associated with reduced function of sy-
temic virus-specific CTL, although virus load and plasma cytokine levels were not examined. When taken together, these studies suggest that gonococcal cervicitis may be associated with both systemic Th2 immune activation and with Th1 inhibition. Potential mechanisms were not investigated in the current study; however, Th2 responses are important in immune defense against bacterial pathogens, and IL-4 and IL-10 are the key Th2 effector cytokines [7]. Because the development of Th1 responses can be antagonized directly by IL-4 and indirectly by IL-10 [7], cellular immune responses important in the control of persistent viral infections, including CMV and HIV-1 infection, might be impaired as a consequence of an effective antibacterial immune response. However, confirmation of this hypothesis will require the simultaneous examination of both Th1 and Th2 immune markers in the setting of incident bacterial infections. Interestingly, the reduced cellular immune control of HIV-1 infection associated with gonorrhea might contribute to the rapid disease progression seen in this sex worker cohort after acute HIV-1 infection [15].

HIV-1–specific responses in HEPS sex workers fall below the level of detection of tetramer studies or intracellular cytokine staining; thus, function of HIV-1 epitope–specific CD8+ T cells in HEPS women could not be examined using these techniques. However, the loss of HIV-1–specific ELISPOT responses associated with gonococcal cervicitis, as well as the demonstration of diminished function within CMV-specific CD8+ T cells, suggests that cervicitis was also associated with reduced function in HIV-1–specific CD8+ cells. Impaired function of protective HIV-1–specific CTL, as a consequence of gonococcal cervicitis in HEPS women, therefore may provide a mechanism by which nonulcerative STDs enhance HIV-1 susceptibility [2].
In this study, gonococcal cervicitis was associated with diminished HIV-1– and CMV-specific CD8+ lymphocyte function. A better understanding of the relationship between mucosal infections and systemic immunity will be useful in developing strategies to reduce HIV-1 genital shedding and disease progression in seropositive women and HIV-1 susceptibility in those at risk of infection.

Acknowledgments

We thank the women of the Pumwani clinic (Nairobi) for their patience and cooperation throughout the study; Erastus Irungu and Julius Oyugi for technical assistance; Jane Kamene Musyoka for assistance in enrollment and follow-up of study subjects; and Kati di Gleria for peptide synthesis.

References