Mucosal and Systemic Immune Activation Is Present in Human Immunodeficiency Virus–Exposed Seronegative Women

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Immune parameters were analyzed in peripheral blood mononuclear cells (PBMC) and cervical mucosal biopsy specimens of human immunodeficiency virus (HIV)–seronegative women sexually exposed to HIV (exposed seronegative [ESN]), HIV-infected women, and healthy women without HIV exposure. HIV was not detected in PBMC or cervical mucosal biopsy specimens of ESN women. However, interleukin (IL)–6, IL-10, IL-12, interferon (IFN)–γ, and tumor necrosis factor (TNF)–α and –β mRNA were elevated in PBMC and cervical mucosal biopsy specimens of ESN and HIV-infected women; CCR5 and CXCR4 mRNA were augmented in cervical mucosal biopsy specimens, but not in PBMC, of ESN and HIV-infected women; HIV-specific IFN-γ–secreting cells were detected in vaginal washes of ESN and HIV-infected women; and phenotypic alterations were present in PBMC of ESN women. These results suggest that active HIV infection is not required for T cell activation; immune alterations occur in women in whom HIV infection cannot be detected virologically or clinically.

In recent years, different subject cohorts have been investigated in whom exposure to human immunodeficiency virus (HIV) did not result in clinical infection [1–3]. Resistance to HIV infection in exposed seronegative (ESN) persons is associated with immunologic parameters, such as type 1 cytokine production by HIV-stimulated peripheral blood mononuclear cells (PBMC) [4–6]. HIV-specific and CD8+ cytotoxic T lymphocyte (CTL)–mediated cytotoxicity [7–12], β-chemokine production by HIV-stimulated PBMC [13]. HIV-specific mucosal [14–17] and serum [18] IgA, and mucosal CD8+ lymphocyte–mediated interferon (IFN)–γ responses to HIV peptides [19]. Resistance to HIV infection is also associated with mutations in the CCR5 coreceptor [20, 21]; different CCR5 mutations confer total or partial resistance to infection by HIV R5 strains in vitro and delay disease progression in vivo [22, 23]. Exposure to infection with defective viral strains is postulated as a possible explanation for this phenomenon.

Resistance to HIV could be associated with (possibly repeated) aborted infections that the immune response is able to ward off or, alternatively, could be correlated with low-level HIV infection that might not be detectable with current virologic methods. The most likely hypothesis is that resistance to HIV infection is a phenomenon that includes both of these possibilities. On one hand, data showing that HIV-specific humoral and cell-mediated responses are lost once exposure to the virus is interrupted indicate that actual subclinical HIV infection is not taking place in ESN subjects [5, 14]. On the other hand, the likelihood that low-level infection does take place in some of these persons is suggested by the fact that elicitation of a CTL response is secondary to viral replication within the host’s cells and presentation of viral antigens in association with HLA class I molecules [7–12]. Nonetheless, HIV has never been detected in PBMC of ESN persons.

Among discordant heterosexual couples who do not habitually practice safe sex, HIV exposure would most likely take place in the mucosal genital tract. This raises the possibility that virus exposure could result in a peculiar and possibly compartmentalized immune response and that HIV could be detected in genital mucosal tissues. To study mucosal immunity in seronegative HIV exposure in depth, we analyzed cytokine mRNA, the expression of HIV coreceptors, and the presence of gp160 peptide–specific CD8+– and IFN-γ–producing lym-
phocytes in cervical mucosa biopsy specimens of ESN, HIV-seropositive, and HIV-seronegative women at low risk of infection. The same parameters, along with the expression of a panel of immunophenotypic markers, were examined in PBMC of all subjects to verify whether exposure to HIV results in clearly distinct and compartmentalized immune responses.

Subjects and Methods

Study population. Nine heterosexual couples discordant for HIV serostatus were enrolled in the study. All men were infected with HIV, and all women were HIV seronegative despite a prolonged history of penetrative sexual intercourse without condoms (and no other known risk factors). The inclusion criterion for the ESNs was a history of multiple unprotected sexual episodes (with the same HIV-seropositive partner) for ≥4 years, with at least 1 episode of at-risk intercourse within the 4 months before study entry. Of the 9 HIV-seropositive partners, 5 had been exposed to HIV via injection drug use and 4 sexually (2 bisexuals and 2 heterosexuals). Nine age-matched HIV-infected women were also enrolled in the study (2, 1993 CDC class A1; 3, A2; and 4, C3). Eight of the 9 HIV-infected subjects had been receiving antiretroviral therapy (4, 2 nucleoside reverse-transcriptase inhibitors [NRTIs]; 4, 2 NRTIs and 1 protease inhibitor) for ≥1 year at the time of the study; CD4 cell counts were 317±715, and HIV plasma viremia was >400 copies/mL in 5 of the 9.

Four age-matched healthy women volunteer controls (HCs) undergoing a routine Pap test and without any known risk factor for HIV infection were studied as well. All women underwent careful gynecologic and laboratory evaluation; results of the evaluation did not any reveal concomitant infectious or gynecologic problems. All women (ESNs, HIV seropositives, and HCs) had been longitudinally followed for ≥3 years before the study by the Department of Obstetrics and Gynecology, Santa Maria Annunziata Hospital, Florence. This allowed us to exclude from the study ESNs and HCs in whom sexually transmitted diseases or any other oncologic or gynecologic pathology was reported during that time period. The ESN subjects were characterized on the basis of the presence of CCR5 Δ32 alleles; a heterozygous deletion was detected in 1 of 9 subjects. All 9 ESNs and low-risk uninfected healthy women agreed to donate PBMC and to undergo multiple cervical biopsies.

In contrast, although all HIV-infected patients donated blood, cervical biopsies were possible only in 4 of 9 patients. Four different biopsy mucosal specimens were collected from all women study participants: 1 specimen from the anterior fornix, 1 from the posterior fornix, and 2 cervical mucosa biopsy specimens. Biopsy tissues were placed in a Nalgene vial containing 1 mL of PBS (Organon Teknika, Durham, NC) and were immediately deep frozen in a liquid nitrogen tank. After study enrollment, counseling was offered to all couples.

Blood drawing and processing. Whole blood was collected by venepuncture in EDTA-containing vacutainer tubes (Becton Dickinson, Rutherford, NJ). After PBMC were separated on lymphocyte separation medium (Organon Teknika) and washed twice in PBS, the number of viable leukocytes was determined by trypan blue exclusion.

RNA extraction. Total RNA was extracted from lymphocytes and biopsy specimens by the acid guanidin thiocyanate-phenol-chloroform method. The purity of the extracted RNA was determined by spectrophotometry. Extracted RNA was treated with RNase-free DNase (RQI DNase; Promega, Madison, WI) to remove contamination of genomic DNA [24, 25].

Reverse transcription. We reverse transcribed 1 μg of total RNA from lymphocytes and from biopsy specimens into first-strand cDNA in a 20-μL final volume containing 1 μM of random hexanucleotide primers, 1 μM of oligo dT, and 200 U of Moloney murine leukemia virus reverse transcriptase (RT; Clontech, Palo Alto, CA).

Determination of virus load. To quantify HIV virus load, we used an RNA exogenous competitor in competitive RT–polymerase chain reaction (PCR; Shuttle Biotech, Milan, Italy). For competitive analysis, the 1551–1666 gag fragment of the HIV-1 genome (highly conserved and amplified by the sensitive and widely used SK38/SK39 primer set) was selected. This method is highly sensitive (10 copies of HIV RNA/mL) [26, 27].

Normalization of sample β-actin cDNA content by competitive PCR. To compare cytokine mRNA expression in the different samples, it was essential to use equivalent amounts of substrate cDNA. Thus, all samples were normalized for β-actin cDNA content by competitive PCR (Clontech). In brief, 1 set of primers (5'-ATCGGCACACACTTCTACAATGAGCTGG-3' and 5'-CGTCATACTCTGGCTGTGATCCACATCG-3') amplified both β-actin cDNA in samples and competitor cDNA. The PCR products of both target and competitor were subjected to acrylamide gel electrophoresis. The competitor cDNA generated a shorter PCR product (619 bp) than did the target cDNA (838 bp). Densitometry (INTAS, Göttingen, Germany) was used to quantitate the density of the bands of samples and competitor PCR products. The concentration of substrate sample cDNA was calculated from the concentration of competitor cDNA that generated equal amounts of competitor and sample products. Therefore, by plotting the ratio of sample density to competitor PCR product against the known amount of competitor substrate cDNA, the amount of substrate β-actin cDNA in each sample was calculated. To normalize the cDNA sample concentration, all samples were diluted to the same concentration as the sample with the lowest cDNA concentration.

Quantification of cytokine cDNA by PCR analysis. Each PCR analysis was performed in a 50-μL reaction mixture containing 10 μL of RT reaction mixture, 1X PCR buffer (20 mM Tris HCl, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40, and 50% glycerol), 200 μM concentration of each dNTP, 1.25 U of Taq polymerase (Takara, Otsu, Japan), 0.4 μM of β-actin primers, and 0.4 μM for each cytokine (interleukin [IL]-2, -4, -6, -10, and -12; tumor necrosis factor [TNF]-α and −β, and IFN-γ). We did thermal cycling (Touchdown Hybaid; Celbio, Pero, Italy) with the following amplification profile: initial denaturation, 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 s; annealing at 58°C for 30 s; extension at 72°C for 30 s; and a final amplification step at 72°C for 10 min. The PCR reaction products were then electrophoresed in a 10% acrylamide gel and stained with 0.5 μg/mL ethidium bromide. The size of each cDNA product was determined by comparison with a DNA size marker (pBR322; Sigma, St. Louis). To quantify relative levels of gene expression, gel bands were scanned by transmission densitometry,
and the areas of the peaks were calculated in arbitrary units. To evaluate the relative levels of expression of the target genes in RT-PCR, the value of the internal standard (β-actin) in each test tube was used as the baseline gene expression of that sample; relative values were calculated for each of the target genes amplified in that reaction. These values were then used to compare expression across samples tested.

Quantification of CCR5 and CXCR4 cDNA by competitive PCR. To quantify the expression of CCR5 and CXCR4, we used an exogenous competitor in competitive PCR (Maxim Biotech, San Francisco). The competitor and the template have similar lengths and the same primer recognition sequences, thus ensuring identical thermodynamics and amplification efficiency for both template species. The amount of the competitor is known. After amplification, products of both templates are distinguished by gel electrophoresis to allow densitometric evaluation of the relative intensities of the bands. The ratio of amplification products reflects the ratio between the initial amount of the template, thus allowing the precise evaluation of CCR5 and CXCR4 cDNA amounts.

IFN-γ ELISPOT assays. IFN-γ ELISPOT analyses were performed on cervical samples of 5 ESN, 5 HIV-seropositive, and 4 HC women. Cervical samples were obtained, as described elsewhere [19], by using a cytobrush (Histobrush; Spectrum Labs, Dallas), which was inserted in the cervical os and rotated 360°, and the sample thus obtained was transferred immediately into 5 mL of RPMI. The cytobrush specimen was not collected from menstruating women and was not utilized if it contained visible blood. The cytobrush was agitated and discarded, and the remaining cell suspension was once more agitated to loosen clumps of mucosa. Cervical mononuclear cells (CMC) were then isolated according to the protocol used for PBMC and were resuspended in RPMI with 2% AB serum. To detect peptide-specific IFN-γ release by PBMC or CMC, 96-well nitrocellulose plates were precoated with a first layer of IFN-γ monoclonal antibodies (Mabs; Mabtech, Nacka, Sweden). PBMC or CMC were then added in duplicate wells, either with a pool of 5 synthetic peptides from the HIV-I gp160 envelope, as described elsewhere [20] (20 μM final concentration env), in the presence or absence of neutralizing anti-CD4 MAb (see below); with no peptide (negative control); or in 1:100 phytohemagglutinin (M form; positive control; Sigma). The 5 peptides used in the stimulation (env T1, env T2, env Th4, env P18IIIIB, and env p18MN) are based on the sequence of gp160 from HIVun or HIVes, and were predicted to be immunogenic because of their amphipathic helical folding potential [29]. The peptides are promiscuous and are recognized by multiple HLA class I molecules (including HLA-A1, -A2, and -B8). HLA-A2′ and -A2′ PBMC lysed HLA-A1′, -A2′, and -B8′ and -DR2′-matched targets pulsed with peptides T1, Th4, P18IIIIB, and P18MN (but not targets matched only for DR2). These peptides have been described in detail elsewhere [30].

Because these epitopes can also be recognized by HLA class II molecules [28], IFN-γ production by CD4 cells was blocked by preincubating CMC and PBMC with 100 ng/mL of neutralizing recombinant human CD4 MAb (R&D Systems, Minneapolis). PBMC assays were run at 2 × 10⁶ and CMC at 5 × 10⁶ cells/well. Plates were incubated overnight at 37°C in 7% CO₂, then the cells were discarded, and the plates were incubated at room temperature for 3 h. We subsequently used a second biotinylated anti-IFN-γ MAb (7-B61-biotin; Mabtech), followed by streptavidin-conjugated alkaline phosphatase (Mabtech) for 2 h. Individual IFN-γ–producing cells were detected as dark blue spots by using an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA). The spots were visually counted, and numbers were confirmed by use of a 40× dissecting microscope. HIV-specific responses were reported as the number of spot-forming units (sfu)/10⁶ PBMC after subtracion of background IFN-γ secretion. We report only responses observed in the wells in which IFN-γ production by CD4+ MAb was blocked by preincubation of CMC and PBMC with the anti-CD4 MAb. A positive response was defined as >20 HIV-specific sfu/10⁶ cells and HIV-stimulated sfu exceeding background by a factor >2.

Immunophenotype analyses. Lymphocyte subsets were evaluated by flow cytometer (Epics XL; Coulter Electronics, Miami Lakes, FL), using 100 μL of EDTA peripheral blood incubated 30 min at 4°C with fluorochrome-labeled Mabs. Erythrocyte lysis was obtained after incubation with an Immuno-Prep Epics Kit and a Q-prep Work Station (both Coulter Electronics). Lymphocytes were selectively analyzed, using forward and side-scatter properties. For each sample, multiparametric data were acquired for 5000 events.

Statistical analyses. Statistical analyses were based on a nonparametric Jonckheere-Terpstra test for trends. All data were also analyzed by a nonparametric Kruskal-Wallis test. In the analysis of immunophenotypic responses, the differences between the mean values of different groups were calculated for each variable by multiple linear regression with dummy variables adjusted for CD4 and CD8 numbers. All P values are 2 sided.

Results

HIV infection is not detected in PBMC or genital mucosa of ESN and HC subjects. The presence of HIV-1 genetic material (1551–1666 gag fragments of the HIV-1 genome) was analyzed by an ultrasensitive PCR method [26, 27] in PBMC of 9 ESNs, 9 HIV-infected women, and 4 HCs and in genital mucosa biopsy specimens of all ESNs, 4 HIV-infected women, and 4 HCs. To optimize the possibility that HIV infection could be detected, 4 different biopsy specimens were analyzed from each ESN and HC (36 biopsy specimens from ESNs and 16 from HCs). Results demonstrated the presence of low amounts of HIV genome (median, 105 copies/mL) in PBMC and mucosal specimens of all HIV-infected persons who were undergoing antiretroviral therapy at the time of the study. In contrast, HIV infection was not detected in PBMC of mucosal specimens of ESNs or HCs (data not shown).

Cytokine mRNA in PBMC and genital mucosa. The mRNA for a panel of cytokines (IL-2, IFN-γ, IL-4, -6, -10, and -12 and TNF-α and -β) was analyzed in PBMC of all ESNs, HIV-infected patients, and HCs and in genital mucosa biopsy specimens of all ESNs, 4 of 9 HIV-infected women, and all HCs. Representative results in PBMC and mucosa biopsy specimens from 1 ESN, 1 HIV-seropositive patient, and 1 HC are shown in figure 1 and figure 2, respectively. In both figures, upper panels illustrate data from an ESN, middle panels show results
for an HIV-infected woman, and lower panels show data for a HC. As shown in figure 1, expression of mRNA for IL-6 and -10, IFN-γ, and TNF-α and -β was detected in PBMC of an ESN (upper panel) and an HIV-seropositive woman (middle), but not in PBMC of an HC (lower panel), in whom mRNA for all cytokines was marginal. IL-12 mRNA was observed in PBMC of an ESN but was very weak in the cells of HIV-infected women and HCs.

Figure 2 shows a similar pattern in the genital mucosa biopsy specimens. Thus, mRNA for IL-6 and -10, IFN-γ, and TNF-α and -β was expressed in mucosal tissues of an ESN (upper panel) and an HIV-infected woman (middle panel), but not in specimens from an HC (lower panel). Again, IL-12-specific mRNA was observed in ESNs, but not in HIV-infected persons or in the HC.

Results from all specimens (median ratio between cytokine-specific mRNA/β-actin) are summarized in figure 3 (mucosal biopsy specimens and PBMC). Marginal expression of IL-2-specific mRNA was detected only in PBMC of ESNs. IL-4 mRNA was conspicuously expressed by PBMC of HIV-seropositive women, but expression of IL-12 mRNA was significant only in ESNs (in whom it was detected in both PBMC and mucosa biopsy specimens), and IFN-γ-specific mRNA was expressed more in ESNs than in HIV-seropositive subjects, both in PBMC and in mucosal specimens. There was an abundance of mRNA for IL-6 and -10 and TNF-α and -β in PBMC and genital mucosa biopsy specimens of both ESN and HIV-seropositive women. However, mRNA for all cytokines was only marginally expressed in HCs. These data indicate that, with the remarkable exception of IL-12, which is mostly detected in ESNs, the cytokine profile pattern of ESN subjects is more similar to that observed in HIV-infected women than to that characteristic of HCs. Thus, immune activation is present in ESNs.

HIV mRNA coreceptors in PBMC and genital mucosa. mRNA for CCR5 and CXCR4, the 2 main HIV coreceptors, was analyzed in PBMC of all ESNs, HIV-infected patients, and HCs and in genital mucosa biopsy specimens of all ESNs, 4 of 9 HIV-infected women, and all HCs. Analysis of HIV coreceptors would have been more indicative if performed on purified CD4 T cells. Nevertheless, we decided to study mRNA coreceptors in whole PBMC because of the difficulty in purifying cell subpopulations from tissue biopsy specimens and to allow the comparison of results of analysis of PBMC and mucosa biopsy specimens. As shown in figure 4, in PBMC of all subjects, CXCR4-specific mRNA was prevalent, in comparison
Figure 3. Median cytokine-specific:β-actin ratios in genital mucosa biopsy specimens (top) and peripheral blood mononuclear cells (PBMC) (bottom) of human immunodeficiency virus (HIV)-exposed seronegative women (ESNs), HIV-infected patients (HIV seropositives), and healthy controls (HIV seronegatives). Bars indicate SDs. * $P < .05$; ** $P < .005$. IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

with CCR5-specific mRNA; mRNA for both chemokine receptors was similar in PBMC of HIV-seropositive subjects, ESNs, and HCs (left and middle panels). Also, both CCR5- and CXCR4-specific mRNA were augmented in genital mucosa biopsy specimens of HIV-seropositive subjects and ESNs, in comparison with HCs (right panel).

**HIV-specific ELISPOT responses in PBMC and vaginal washes.** We analyzed env peptide–specific IFN-γ–secreting CD8 T cells in a subgroup of ESNs ($n = 5$), HIV-seropositives ($n = 5$), and HCs ($n = 4$); both cells derived from vaginal washes (CMC) and PBMC were examined upon stimulation with a pool of gp160 peptides recognized by multiple HLA class I molecules (including HLA A1, A2, and B8) [30]. Because these epitopes can also be seen by HLA class II molecules, IFN-γ production by CD4 cells was blocked by preincubating CMC and PBMC with a CD4-neutralizing MAb. HIV-specific and IFN-γ–secreting cells (≥2-fold above background) were detected in PBMC of 2 of 5 HIV-seropositive women but in
neither ESNs nor HCs. In contrast, when vaginal washes were examined, HIV-specific and IFN-γ-secreting cells were detected in 3 of 5 ESNs (and borderline positivity in another ESN), in 4 of 5 HIV-seropositive women, and in 0 of 5 HCs (figure 5).

Although no statistical difference was detected when HIV-specific and IFN-γ-secreting cells were compared in CMC of ESN and HIV-infected women, these cells were significantly more frequent in CMC of both ESN and HIV-infected women than in CMC of HCs (P = .007 and .001, respectively).

**Immunophenotypic analyses on PBMC.** The expression of a panel of immunophenotypic markers was evaluated on the surface of PBMC from all ESN, HIV-seropositive, and HC subjects. Significant differences are shown in figure 6. The following differences emerged in the CD4 lymphocyte subpopulation. First, CD4+/45RA+/62+ lymphocytes were diminished in both ESN and HIV-seropositive subjects, compared with HC subjects (ESN vs. HC, P = .069; HIV seropositive vs. HC, P = .01). Second, CD4+/45RO+ lymphocytes were augmented in ESNs and HIV-seropositive women, compared with HCs (ESN vs. HC, P = .001; ESN positive vs. HC, P = .006), and, as a consequence, the memory:naïve ratio was altered (ESN = 4.62; HIV seropositive = 3.67; HC = 2.98). Third, CD4+/CD25+ lymphocytes were augmented in ESNs, compared with both HIV-seropositive and HC subjects (ESN vs. HC, P = .006; ESN positive vs. HC, P = .007). Fourth, CD4+/CD28- lymphocytes were augmented in ESNs and HIV-seropositive women, compared with HC subjects (ESN vs. HC, P = .007). There were also significant differences when subpopulations of CD8+ T lymphocytes were examined: CD8+/CD28+ T lymphocytes were augmented in ESN and HIV-seropositive women, compared with HCs (ESN vs. HC, P = .006; HIV seropositive vs. HC, P = .01), whereas CD8+/CD28- lymphocytes were increased in HIV seropositives but not in ESNs. In addition, CD8-CD38+ T lymphocytes were aug-
Figure 6. Expression of panel of immunophenotypic markers on surface of peripheral blood mononuclear cells (PBMC) of human immunodeficiency virus (HIV)-exposed seronegative women (ESNs), HIV-infected patients, and healthy controls (HCs). Values are median ± SD.

Discussion

The vast majority of new worldwide HIV infections are sexually acquired [31], and the genital mucosa is the first target for HIV by this transmission route [32–35]. We recently showed that HIV-specific IgA antibodies are present in the cervicovaginal secretions (CVSs) of sexually exposed women who do not become infected (ESNs) [14]. These findings were confirmed by analyses of Kenyan [15] and Thai [16] HIV-resistant sex workers. The IgA observed in CVSs of ESNs is present in high titers [14], recognizes conformational epitopes on gp41 that differ from those seen by IgA of HIV-seropositive persons [17], and is capable of neutralizing in vitro primary HIV isolates [17].

In an attempt to better characterize mucosal immunity in ESNs, we analyzed a panel of immune parameters in CVS biopsy specimens and in CVS washes and specimens from HIV-seropositive and -negative controls (HCs) and then compared the results with those obtained in PBMC. We found elevated expression of mRNA for IL-6, IL-10, IFN-γ, and TNF-α and -β in genital biopsy specimens and PBMC of ESNs and HIV seropositives but not in those of HCs; high expression of CCR5- and CXCR4-specific mRNA in genital biopsy specimens, but not in PBMC, of both ESN and HIV-seropositive persons; and HIV-specific IFN-γ-secreting CD8 T lymphocytes in CVS washes (but not in PBMC) of both ESN and HIV-seropositive women. In addition, we found a phenotypic pattern of immune activation in PBMC of ESN and HIV-seropositive women. These data indicate that immune activation characterizes ESNs in the absence of any detectable HIV infection. ESNs are, therefore, apparently immunologically more similar to HIV-infected persons than to HCs.

Epidemiologic and immunologic observations support the possibility that the immune activation observed in ESNs is associated with exposure to HIV. Thus, all the women (ESNs, HIV seropositives, and HCs) selected for the study were enrolled in a longitudinal program of the Department of Obstetrics and Gynecology, Santa Maria Annunziata Hospital, Florence, and had been followed ≥3 years before the study.
period. This allowed us to exclude from the study ESNs and HCs in whom sexually transmitted diseases or other oncologic or gynecologic pathology was reported before the study. All of the women underwent careful gynecologic and laboratory screenings at the time of study entry; results of the evaluations did not reveal concomitant infectious or gynecologic problems. Finally, HIV-specific IFN-γ–secreting CD8 T lymphocytes were observed in the CVSs of the majority of ESNs.

mRNA for cytokines that mediate immune inflammation (IL-6, IFN-γ, and TNF-α and -β) was overexpressed in both PBMC and genital biopsy specimens of ESN and HIV-seropositive subjects. These findings suggest that exposure to HIV results in an immune scenario that is similar to that associated with actual HIV infection and is characterized by immune activation. Immune activation is thought to favor HIV infection, because the virus replicates better inside activated cells [36–40]. The observation that immune activation is detected both in persons in whom infection has taken place and in persons who are seemingly resistant to the virus is in apparent contrast with this statement. On the other hand, both in PBMC and in genital biopsy specimens of ESNs (but not of HIV-seropositive persons), immune activation was associated with increased amounts of the type 1 and cell-mediated immunity–inducing cytokines, IFN-γ and (mainly) IL-12. Both IFN-γ and IL-12 stimulate cell-mediated immunity (CMI), in particular the activity of cytotoxic T lymphocyte and of NK cells [41, 42]. Strong CMI has been hypothesized to be a correlate of immune protection against HIV infection in ESNs [43]. Therefore, augmentation of these cytokines could be potentially associated with apparent resistance to infection. Augmented type 1 cytokine production in the mucosal compartment is also likely to be correlated with the detection of HIV-specific and IFN-γ–secreting CD8 T lymphocytes in the cervix of ESN women. These results confirm the recent data of Kaul et al. [19], who described the presence of mucosal HIV-1–specific CD8+ lymphocyte responses in HIV-exposed seronegative Kenyan sex workers. The presence of HIV-specific and IFN-γ–secreting lymphocytes in the cervix of women who are apparently resistant to HIV infection also reinforces previous results showing that mucosal (but not systemic) HIV-specific CD8+ CTL are associated with long-lasting immune resistance to mucosal virus transmission in mice [44] and that env-specific major histocompatibility complex class I–restricted lymphocytes in the jejunal lamina propria are correlated with protection from challenge with infectious doses of simian immunodeficiency virus [45].

Immunophenotypic analyses suggested that the circulating lymphocytes of ESNs are activated. Thus, the CD4+ CD25+ and CD8+ CD38+ T lymphocytes were significantly augmented in ESNs, compared with HCs. A decrease in CD4+ naive cells and an increase in CD4+ memory lymphocytes that resulted in alteration of the naive:memory ratio was present in both ESNs and in HIV-seropositive patients. This finding suggests that the same immunologic stimulus/signal that leads to prolonged immune activation in HIV-seropositive persons also occurs in ESNs. CD4+ CD28+ cells were increased in ESNs; these cells could be important in antiviral defenses, because they may be IFN-γ–producing and perforin-positive cells that are functionally specialized for killing [46, 47]. The increase in CD8+ CD28+ lymphocytes observed in ESNs is also intriguing, because these cells produce cell antiviral factor [48], a soluble factor that can suppress HIV replication [48, 49]. The observation that CD8+ CD28+ T cells are also elevated in HIV-seropositive persons but not in ESNs could, again, be biologically relevant, because these cells function as T suppressor lymphocytes that can inhibit the proliferative response of antigen-stimulated CD4+ T helper cells [50, 51].

Despite the mostly descriptive nature of this study, we attempted to determine interrelationships among the different immunologic parameters analyzed in ESN subjects. Results showed that ESN women in whom HIV-specific mucosal CD8 T lymphocytes were detected were characterized by the highest expressions of IFN-γ–negative–specific mRNA, as well as the highest percentages of circulating CD4+ and CD8+ memory T lymphocytes. CD8+ 28+ T cells were also particularly elevated in these women.

In contrast to the results summarized, chemokine-specific mRNA was altered (similar to that observed in HIV-infected persons) in mucosa biopsy specimens but not in PBMC of ESNs. β-chemokine receptors are up-regulated upon immune activation [52, 53], and their expression is enhanced by some cytokines (especially IFN-γ and IL-12) [54–56] that are augmented in ESNs. The apparent discrepancy observed when results of cytokine and immunophenotype analyses (mucosal and systemic activation) are compared with those of chemokine receptors and of HIV-specific and IFN-γ–secreting CD8 lymphocytes (mucosal but not systemic activation) might be secondary to the cell types prevalent in the mucosa biopsy specimens. Alternatively, the observation that the mucosa is more immune activated than circulating PBMC might be secondary to a limited, and undetectable, infection with HIV, which is contained by the immune response within the genital mucosa. Against this possibility is the fact that HIV was not observed when ultrasensitive PCR analysis was performed on 36 biopsy specimens from ESNs (HIV was found in all specimens from HIV-seropositive subjects). It remains to be determined whether virologic analyses on more specimens or on serial lymph node biopsy specimens would allow the detection of minimal confined HIV replication within the genital mucosa.

In summary, we found that women exposed to HIV who are apparently resistant to infection have systemic and mucosal immune activation and mucosal IFN-γ–secreting CD8 lymphocytes. These results suggest that immune activation in itself is not sufficient to favor HIV infection, nor is it necessarily an immunologic marker of HIV disease. These results also reinforce the idea that HIV-specific and IFN-γ–secreting lymphocytes in the genital tract may be associated with protection
against heterosexual HIV infection in exposed, uninfected women.

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