MAJOR ARTICLE

HIV-Specific T Helper Responses and Frequency of Exposure among HIV-Exposed Seronegative Female Sex Workers in Abidjan, Côte d'Ivoire

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Background. The characteristics of human immunodeficiency virus (HIV) exposure that determine the induction of HIV-specific T cells and, in particular, T helper cells are not well understood.

Methods. HIV-1 Gag- and Env-specific T helper cells were analyzed by use of an interferon (IFN)–γ enzyme-linked immunosorbent spot (ELISPOT) assay and by use of IFN-γ secretion flow cytometry. Responses among HIV-exposed seronegative (ESN) female sex workers (FSWs) were compared with responses among HIV-seropositive FSWs and HIV-seronegative female blood donors from Abidjan, Côte d'Ivoire.

Results. Low-level ELISPOT responses were detected in 8 (20%) of 40 ESN FSWs. All of 25 HIV-seropositive FSWs had high-level ELISPOT responses. HIV-specific $CD4^+$ T cells and, occasionally, $CD8^+$ T cells were detected by secretion flow cytometry in 3 (38%) of 8 ESN FSWs and in 4 (80%) of 5 HIV-seropositive FSWs. ESN FSWs with detectable HIV-specific T helper responses had more clients on the previous working day ($P = .02$) and more exposures to HIV per month ($P = .02$) and tended to have a lower total duration of commercial sex work.

Conclusions. These findings demonstrate that the presence of HIV-specific T helper cells in ESN FSWs is associated with the frequency, rather than the duration, of exposure to HIV. The data may have important implications for the evaluation of HIV vaccine efficacy.

Some individuals appear to resist HIV infection, despite frequent exposure to the virus [1–4]. These individuals constitute an ideal group for studying the mechanisms of protection against HIV infection. To date, HIV-specific $CD8^+$ cytotoxic T cells (CTLs) probably are the best-documented correlate of HIV protection in HIV-exposed seronegative (ESN) subjects, because they have been detected in several populations by use of a variety of techniques [2, 5–9]. Furthermore, a positive association has been found between the occurrence of HIV-specific CTLs and the duration of prior sex work among ESN female sex workers (FSWs) in Nairobi [10, 11]. Unfortunately, a number of these ESN FSWs became HIV-seropositive despite preexisting CTL responses. This phenomenon was found to be associated with a break in sex work or a reduction in the number of clients per day, which could have led to a waning of CTL responses [12]. Although crucial for the design of a protective HIV vaccine, the characteristics of exposure to HIV that are associated with the establishment of HIV-specific immunity in ESN subjects remain largely unknown.

Several reports have consistently demonstrated the importance of the role played by HIV-specific $CD4^+$ T helper cells in the induction and maintenance of host anti-HIV immunity, in particular of HIV-specific CTLs, in HIV-seropositive subjects [13–16]. In agreement with the results of those studies, one can anticipate an essential role for T helper cells in establishing an HIV-protective immune response in ESN subjects. Several studies have indeed reported the presence of HIV-spe-

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cific T helper cells in ESN subjects [1, 4, 7, 17–20]; however, these studies used methods based on lymphocyte proliferation or bulk cytokine secretion that lack the specificity of more recent methods, such as ELISPOT and cytokine flow cytometry assays.

ESN FSWs studied in Abidjan, Côte d'Ivoire, were previously shown to have detectable anti-HIV IgA antibodies in cervicovaginal secretions [21], together with decreased levels of CXCR4 expression and increased levels of T cell activation [22]. In the present study, for the first time to date, peripheral HIV-specific T helper responses were measured in ESN FSWs, by use of the ELISPOT assay and by use of IFN-γ secretion flow cytometry. In addition, we verified whether HIV-specific responses in ESN FSWs were associated with estimations of sexual exposure to HIV, on the basis of the duration of commercial sex work, the number of clients on the previous working day, and the consistency of condom use.

SUBJECTS, MATERIALS, AND METHODS

Study population. During the months of May 2000, February and March 2001, and October 2001, 40 HIV-seronegative and 25 HIV-seropositive FSWs were enrolled consecutively as part of an ongoing surveillance study at a confidential clinic in Abidjan [23]. Blood samples were obtained, and information on sociodemographic characteristics and sexual behavior were obtained during a personal interview by use of a standard questionnaire. None of the HIV-seropositive FSWs had received antiretroviral therapy before enrollment. Blood samples were also obtained from 32 HIV-seronegative female blood donors at the national blood transfusion center in Abidjan. The study was approved by the institutional review board of the Centers for Disease Control and Prevention (Atlanta) and by the ethical committees of the Ministry of Health (Côte d'Ivoire) and the Institute of Tropical Medicine (Antwerp, Belgium). Informed consent was given by all study subjects before enrollment.

Laboratory methods. All laboratory analyses were performed in Abidjan with freshly obtained whole-blood samples. Whole blood was drawn from FSWs and female blood donors into EDTA tubes (Becton Dickinson). The plasma HIV serostatus of all subjects was determined by use of an HIV testing algorithm based on a combination of ELISAs [24, 25]. The HIV serostatus of seronegative FSWs was confirmed by use of HIV-1 reverse-transcriptase polymerase chain reaction [26].

Interferon (IFN)–γ ELISPOT assay. Ninety-six–well polyvinylidene fluoride–bottom plates (Millipore) were coated with 10 μg/mL anti–IFN-γ monoclonal antibody (MAb; Mabtech) overnight at 4°C. Plates were washed with RPMI 1640 medium, blocked with 10% human serum for 1 h, and washed again. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood within 4 h of collection and were resuspended in RPMI 1640 medium containing 5% human serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Roche Diagnostics GmbH). As a negative control, 5×10^5 PBMCs were added to duplicate wells with medium alone. As a positive control, 2.5×10^5 PBMCs were added to duplicate wells with 0.5 μg/mL phytohemagglutinin (PHA; Murex). Baculovirus-expressed HIV-1 proteins Gag p24 (10 μg/mL; EVA620; National Institute for Biological Standards and Control) and Env gp120 proteins (10 μg/mL; EVA607; National Institute for Biological Standards and Control) were added to 5×10^5 and 2.5×10^5 PBMCs, respectively, in 2 wells each of quadruplicate wells. HIV-1 clade B proteins were used, because recombinant proteins from clade A or clade A/G, the predominant HIV-1 strains in Côte d'Ivoire [27], were not available at the time the study was initiated. The addition of 10 μg/mL cytomegalovirus (CMV) lysate (BioWhittaker Europe) served as an additional positive control for the measurement of HIV-specific T cell responses. Plates were incubated overnight at 37°C in 5% CO_2, washed with PBS containing 0.05% Tween 20 (Sigma), and incubated with 1 μg/mL biotinylated anti–human IFN-γ (Mabtech) for 3 h. Plates were washed and incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 2 h, washed again, and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates (Bio-Rad) for 30 min, followed by rinsing in tap water.

Spots were counted manually with a dissecting microscope. Average numbers of spots per well were normalized to spot-forming cells (sfcs)/10^6 PBMCs. Negative-control sfcs were subtracted from antigen-stimulated sfcs, and negative values were scored as zero. HIV protein ELISPOT responses in ESN and HIV-seropositive FSWs were considered to be positive when the following conditions were met: (1) IFN-γ production was present in PHA-stimulated wells; (2) the number of spots in stimulated wells was at least twice that in negative-control wells; and (3) the negative control–subtracted response exceeded a negative cutoff value of 6 sfcs/10^6 PBMCs. The negative cutoff value was calculated as the average HIV protein response plus 3 times the SD among 32 HIV-seronegative female blood donors at lower risk of HIV infection; values of 6 sfcs/10^6 PBMCs were thus obtained for both p24 and gp120 proteins. For gp120, 1 blood-donor sample was excluded in this analysis, because it had high negative-control and gp120 responses. This strategy allowed increased ELISPOT sensitivity, compared with previous studies that used preset cutoff values of 20 or 50 sfcs/10^6 PBMCs [8, 28], and was justified by low numbers of sfcs in negative-control wells for all study subjects and low HIV protein responses among HIV-seronegative female blood donors. CMV lysate ELISPOT responses in all subjects were considered to be positive when conditions 1 and 2 described above were met. Examples of ELISPOT wells for 1 HIV-seropositive FSW are shown in figure 1*A*.

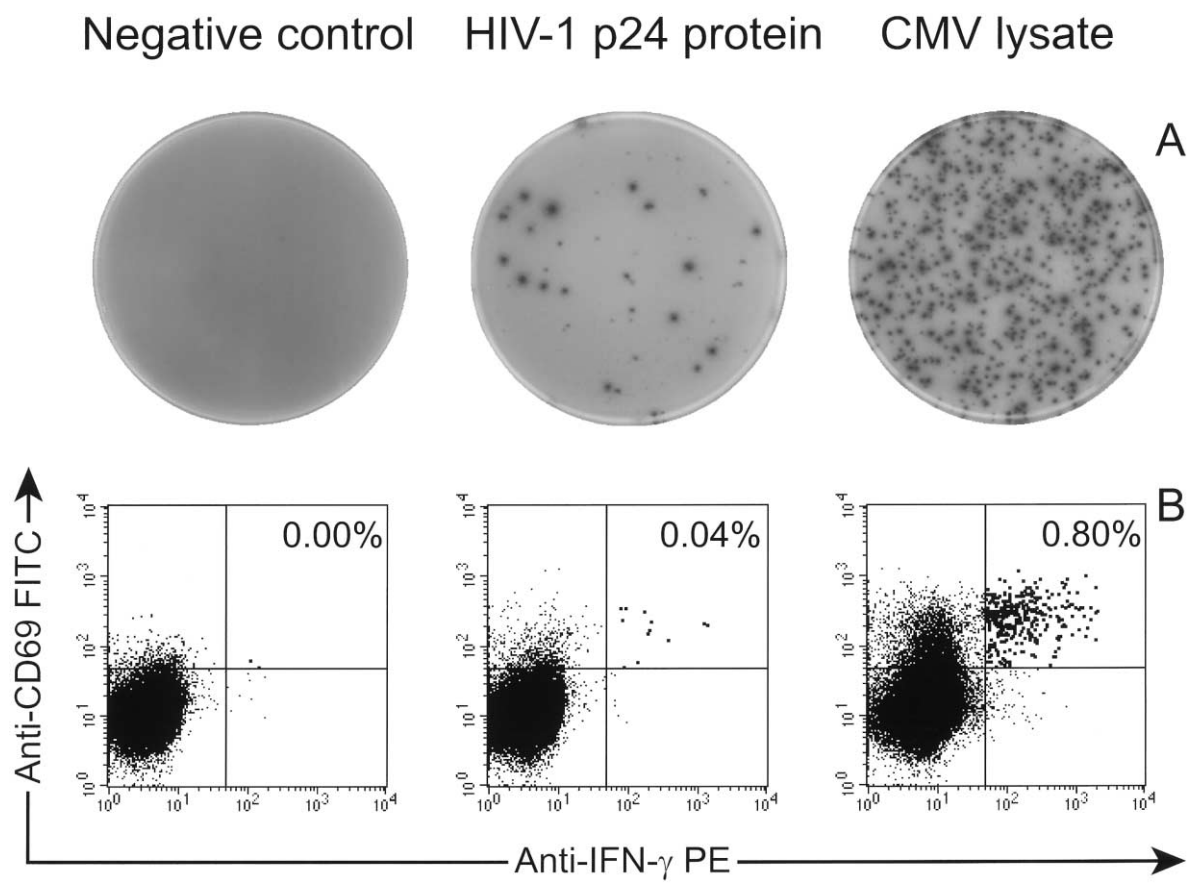

Figure 1. Analysis of antigen-specific T helper responses in 1 HIV-seropositive female sex worker. Presented stimulation conditions are medium alone (negative control), HIV-1 p24 protein, and cytomegalovirus (CMV) lysate (positive control). *A,* Interferon (IFN)–γ ELISPOT assay. *B,* IFN-γ secretion flow cytometry. Dot-plots show gated $CD3^+CD4^+$ (as $CD8^-$) lymphocytes. Percentages of CD69- and IFN-γ–expressing cells in the upper-right quadrant are indicated. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

IFN-γ secretion flow cytometry. IFN-γ secretion flow cytometry was applied to identify HIV-specific $CD4^+$ and $CD8^+$ T cells, according to the manufacturer's instructions (Miltenyi Biotec). In brief, 5×10^6 fresh PBMCs were incubated overnight in 0.5 mL of RPMI 1640 medium containing 5% human serum with medium alone (negative control), 10 μg/mL CMV lysate (positive control), or 10 μg/mL HIV-1 proteins p24 and gp120. The next day, cells were surface-stained with a CD45/IFN-γ bispecific MAb conjugate (Miltenyi Biotec), incubated for 45 min at 37°C to permit surface capture of IFN-γ secretion, and stained with anti–IFN-γ phycoerythrin (Miltenyi Biotec), anti-CD69 fluorescein isothiocyanate, anti-CD8 peridinin chlorophyll protein, and anti-CD3 allophycocyanin fluorochrome–labeled MAbs (all from Becton Dickinson). Cells were analyzed by use of a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson). Gated $CD4^+$ (as $CD8^-$) and $CD8^+$ T lymphocytes coexpressing CD69 and IFN-γ were considered to be antigen specific. Negative-control percentages of $CD69^+$/IFN-γ^+ cells were subtracted from antigen-stimulated percentages, and negative results were scored as zero. HIV-specific T cell responses in ESN and HIV-seropositive FSWs were considered to be positive when (1) percentages of stimulated $CD69^+$/IFN-γ^+ T cells were at least twice the negative-control percentages and (2) negative control–subtracted percentages of $CD69^+$/IFN-γ^+ T cells exceeded negative cutoff values of 0.0090 and 0.0273 for p24-specific $CD4^+$ and $CD8^+$ T cells, respectively, and 0.0106 and 0.0144 for gp120-specific $CD4^+$ and $CD8^+$ T cells, respectively. Negative cutoff values were calculated as the average HIV protein response plus 3 times the SD among 5 HIV-seronegative female blood donors. CMV-specific T cell responses in all subjects were considered to be positive when condition 1, as described above, was met. Examples for the detection of antigen-specific $CD4^+$ T cells are shown for 1 HIV-seropositive FSW in figure 1*B*.

Statistical analysis. The number of exposures to HIV per month was estimated by multiplying the number of clients on the previous working day by the number of days per month, the prevalence of HIV-1 among male clients of FSWs, and the proportion of unprotected sex. The proportion of unprotected sex was based arbitrarily on the reported consistency in using

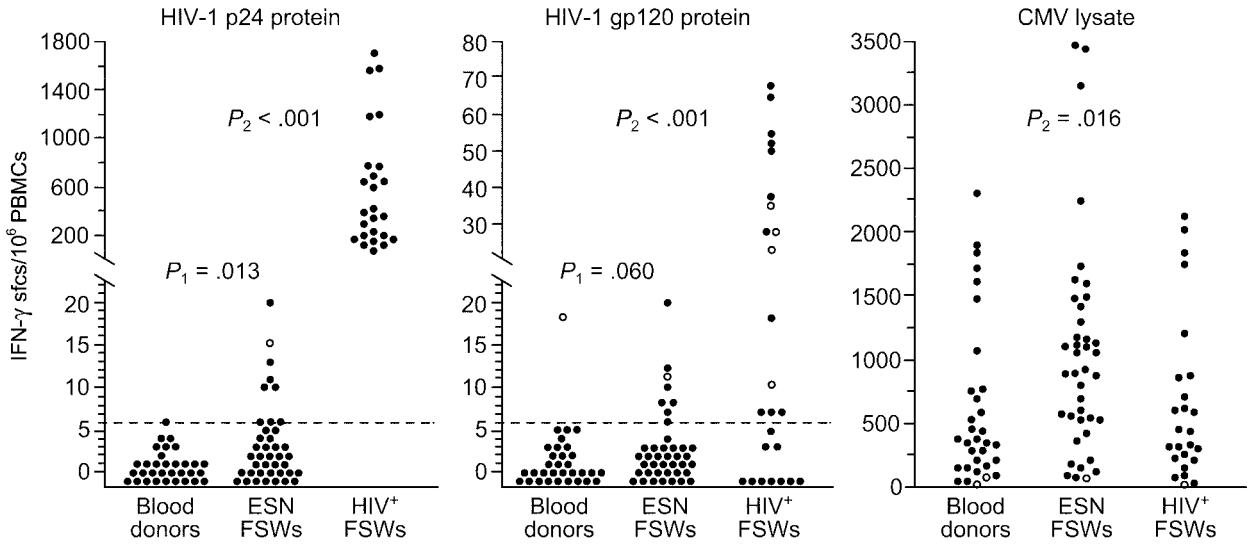

Figure 2. ELISPOT responses for 32 female blood donors, 40 HIV-exposed seronegative (ESN) female sex workers (FSWs), and 25 HIV-seropositive (HIV+) FSWs. Stimulation conditions are HIV-1 p24 protein, HIV-1 gp120 protein, and cytomegalovirus (CMV) lysate. *Dashed lines,* negative cutoff values based on ELISPOT responses among female blood donors (not for CMV lysate); ●, average responses from quadruplicate wells, negative control–subtracted and normalized to nos. of spot-forming cells (sfcs)/10^6 peripheral blood mononuclear cells (PBMCs); ○, responses above the cutoff value that do not exceed twice the negative-control response and are thus considered to be negative. P_1, female blood donors vs. ESN FSWs (Mann-Whitney *U* test); P_2, HIV+ FSWs vs. female blood donors and ESN FSWs (Kruskal-Wallis *H* test).

condoms and was scored as follows: always, 0.1; often, 0.35; rarely, 0.7; and never, 1. In 1998, the prevalence of HIV-1 among male clients of FSWs in Abidjan was 13.4% [29]. The number of lifetime exposures to HIV was derived by multiplying the exposures to HIV per month by the total duration, in months, of commercial sex work. Nonparametric tests were used for statistical analyses. Mann-Whitney *U* and Kruskal-Wallis *H* tests were used for comparing 2 and 3 groups, respectively. The Wilcoxon signed rank test was used for comparing 2 variables in the same group. Spearman's rank test was used for correlations. For all analyses, the level of significance was set at $P \leq .05$.

RESULTS

Characteristics of the study populations. The median age of the 40 ESN FSWs was 22 years (interquartile range [IQR], 21–28 years), compared with 24.5 years (IQR, 21–28 years) for the 32 female blood donors ($P = .319$) and 28 years (IQR, 23–36 years) for the 25 HIV-seropositive FSWs ($P = .01$). The median duration of commercial sex work reported by ESN FSWs was 14 months (IQR, 6–36 months). Thirty-three ESN FSWs (82%) reported using condoms always or often, and 7 (18%) reported using them rarely or never. The median reported number of clients during the previous working day was 3 (IQR, 2–5 clients). ESN FSWs were estimated to have a median of 4.3 unprotected exposures to HIV per month (IQR, 1.7–5.7 exposures to HIV) and a median of 51 lifetime unprotected exposures to HIV (IQR, 18–180 lifetime exposures to HIV). Of 25 HIV-seropositive FSWs, all were seropositive for HIV-1, and 3 (12%) were dually seropositive for HIV-1 and HIV-2.

Detection of low-level HIV-1–specific ELISPOT responses in ESN FSWs. Low-level HIV-1 Env- or Gag-specific ELISPOT responses above the female blood donor cutoff value were found in 8 (20%) of 40 ESN FSWs: in 5 (13%), against HIV-1 p24 protein; in 6 (15%), against HIV-1 gp120 protein; and in 3 (8%), against both p24 and gp120 (figure 2). One additional ESN FSW had p24 and gp120 ELISPOT responses above the cutoff value, but these responses did not exceed twice the number of spots in the negative-control wells and were thus considered to be negative. Regardless of the cutoff value, HIV-1–specific ELISPOT responses were higher among ESN FSWs than among female blood donors, reaching statistical significance for p24 but not for gp120. A direct correlation was found between p24-specific and gp120-specific responses among ESN FSWs ($r = 0.464$; $P = .003$). No differences in magnitude were found between p24-specific and gp120-specific responses among ESN FSWs ($P = .215$).

Detection of high-level HIV-1–specific ELISPOT responses in HIV-seropositive FSWs. High-level ELISPOT responses were detected in all 25 HIV-seropositive FSWs (100%) against Gag p24 protein. Env gp120–specific ELISPOT responses were found in 11 (44%) of 25 HIV-seropositive FSWs, and the magnitude of these responses was lower than that for Gag p24 protein ($P < .001$; figure 2). Four HIV-seropositive FSWs had gp120 ELISPOT responses above the cutoff value that did not exceed twice the number of spots in the negative-control wells

Table 1. Detection of HIV-1 protein–specific and cytomegalovirus (CMV)–specific CD4⁺ and CD8⁺ T cells in 5 female blood donors, 8 HIV-exposed seronegative (ESN) female sex workers (FSWs), and 5 HIV-seropositive FSWs, by use of interferon (IFN)–γ secretion flow cytometry and an IFN-γ ELISPOT assay.

	IFN-γ secretion flow cytometry						ELISPOT assay		
	HIV-1 p24		HIV-1 gp120		CMV lysate				
Group, subject	CD4⁺ T cells	CD8⁺ T cells	CD4⁺ T cells	CD8⁺ T cells	CD4⁺ T cells	CD8⁺ T cells	HIV-1 p24	HIV-1 gp120	CMV lysate
Female blood donors									
LBDC00284	0.0038	0.0059	0.0034	0.0000	**0.2583**	**0.0585**	0	0	**1718**
LBDC00296	0.0044	0.0137	0.0031	0.0079	**0.2181**	0.0080	0	0	**142**
LBDC00338	0.0044	0.0138	0.0059	0.0035	**0.0940**	**0.0168**	0	0	**528**
LBDC00340	0.0008	0.0000	0.0066	0.0064	**0.0615**	0.0000	0	0	**152**
LBDC00341	0.0000	0.0000	0.0013	0.0000	**0.0881**	0.0000	6	4	**368**
ESN FSWs									
LPXB06758	0.0000	0.0000	0.0000	0.0004	**0.0393**	0.0055	0	0	**518**
LPXB06760	**0.0125**	0.0099	0.0028	0.0051	**0.0366**	**0.0202**	0	2	**360**
LPXB06762	0.0000	0.0052	0.0000	0.0000	**0.2745**	**0.0278**	**20**	0	**70**
LPXB06765	0.0000	0.0110	0.0000	0.0000	**0.0237**	0.0000	**10**	**8**	**110**
LPXB06768	0.0018	0.0000	0.0000	0.0016	**0.4140**	**0.1058**	0	**20**	**1106**
LPXB06777	0.0026	0.0229	**0.0182**	0.0041	**0.7229**	**0.0681**	0	4	**2256**
LPXB06778	**0.0106**	0.0000	0.0031	**0.0222**	**0.3744**	**0.0386**	**10**	**8**	**1728**
LPXB06786	0.0042	0.0000	0.0102	0.0034	**0.3547**	**0.0635**	0	2	**1302**
HIV-seropositive FSWs									
LPXB06756	**0.0352**	0.0000	0.0000	0.0053	**0.7913**	**0.0205**	**198**	0	**2132**
LPXB06767	**0.0159**	0.0218	0.0054	0.0114	**0.0640**	**0.0511**	**1698**	0	**214**
LPXB06772	**0.0186**	0.0009	0.0080	**0.0341**	**0.1683**	**0.0165**	**128**	**18**	**584**
LPXB06779	0.0000	0.0000	0.0000	0.0000	**0.0502**	**0.1653**	**770**	**10**	**714**
LPXB06782	**0.1383**	**0.1165**	0.0000	0.0000	**0.1970**	**0.0720**	**776**	35	**1744**

NOTE. ELISPOT data are negative control–subtracted spot-forming cells/10^6 peripheral blood mononuclear cells. IFN-γ secretion flow cytometry data are negative control–subtracted percentages of CD69⁺/IFN-γ⁺ cells within CD4⁺ and CD8⁺ T cell subsets. Values meeting criteria for positive responses are in bold type (see Subjects, Materials, and Methods for details).

and were thus considered negative. Clearly, HIV-1–specific ELISPOT responses were significantly higher among HIV-seropositive FSWs than among ESN FSWs and female blood donors for both p24 and gp120. No significant correlation was found between p24- and gp120-specific responses among HIV-seropositive FSWs ($r = 0.153$; $P = .464$).

ESN FSWs had higher CMV-specific ELISPOT responses than did female blood donors and HIV-seropositive FSWs. Stimulation with CMV lysate was included as a positive control for the detection of virus-specific T cell responses. Thirty (94%) of 32 female blood donors, 39 (98%) of 40 ESN FSWs, and 24 (96%) of 25 HIV-seropositive FSWs had detectable CMV-specific ELISPOT responses (figure 2). Significantly higher CMV responses were found among ESN FSWs than among female blood donors and HIV-seropositive FSWs.

Detection of HIV-1– and CMV-specific CD4⁺ and CD8⁺ T cells by use of IFN-γ secretion flow cytometry. To assess the specificity of the ELISPOT responses in ESN FSWs, HIV- and CMV-specific CD4⁺ and CD8⁺ T cells were analyzed by use of IFN-γ secretion flow cytometry for 8 ESN FSWs, 5 HIV-seropositive FSWs, and 5 HIV-seronegative female blood donors, in parallel with ELISPOT analyses (table 1). Three (38%) of 8 ESN FSWs had percentages of HIV-1–specific T cells above the female blood donor cutoff value. However, detection of HIV-specific T cells in ESN FSWs by use of IFN-γ secretion flow cytometry did not correlate with results of the ELISPOT assay: 1 FSW had positive results in both assays, 2 FSWs had negative results in both assays, and 5 FSWs had discordant results. For the 1 ESN FSW who had positive results in both assays, there was a discrepancy in the magnitude of the responses: 0.01% of p24-specific CD4⁺ T cells should correspond to ~50 sfcs/10^6 PBMCs, if one-half of the PBMCs are CD4⁺ T cells, but only 10 sfcs/10^6 PBMCs were detected. In contrast, among 5 HIV-seropositive FSWs, 4 (80%) had HIV-1–specific responses by both IFN-γ secretion flow cytometry and the ELISPOT assay. IFN-γ secretion flow cytometry failed to show HIV-specific T cells in 1 additional HIV-seropositive FSW who tested positive for p24 and gp120 in the ELISPOT assay. All subjects had detectable percentages of CMV-specific CD4⁺ T cells and, in some cases, CD8⁺ T cells, in concordance with the ELISPOT results.

Detection of HIV-specific responses in ESN FSWs was associated with higher numbers of clients on the previous work-

Table 2. Association analysis between the presence of HIV-specific T helper responses and estimates of exposure to HIV among HIV-exposed seronegative female sex workers.

Characteristic	ELISPOT assay			IFN-γ secretion flow cytometry		
	Negative result (n = 32)	Positive result[a] (n = 8)	P[b]	Negative result (n = 5)	Positive result[a] (n = 3)	P[b]
Age, years	22 (20–27)	23 (21–32)	.463	24 (20–35)	21 (20–42)	.881
Duration of sex work, months	15 (9–48)	11 (3–24)	.215	34 (8–72)	8 (5–48)	.655
Proportion of unprotected sex[c]	0.35 (0.1–0.35)	0.35 (0.1–0.35)	.346	0.35 (0.1–0.35)	0.35 (0.35–0.7)	.121
No. of clients on previous working day	3 (2–5)	4 (3–6)	.143	4 (3–4)	6 (6–9)	**.020**
Exposures to HIV per month	4.3 (1.7–5.7)	4.3 (1.8–6.8)	.786	4.3 (1.4–5.7)	12.8 (8.6–17.1)	**.024**
Exposures to HIV in lifetime	51 (17–265)	40 (10–67)	.327	44 (14–328)	68 (64–821)	.297

NOTE. Data are median (interquartile range), except where noted.

[a] Using negative cutoff values based on HIV-seronegative female blood donors.

[b] Mann-Whitney U test. Bold type indicates $P \leq .05$.

[c] The proportion of unprotected sex was based arbitrarily on the reported consistency in using condoms and was scored as follows: always, 0.1; often, 0.35; rarely, 0.7; and never, 1.

ing day and higher numbers of exposures to HIV per month. The detection of HIV-specific T cells by use of either the ELISPOT assay or IFN-γ secretion flow cytometry was compared with estimations of sexual exposure to HIV among ESN FSWs (table 2). Compared with ESN FSWs with a negative ELISPOT result, ESN FSWs with a positive ELISPOT result showed comparable estimated numbers of exposures to HIV and a similar proportion of unprotected sex. However, the total duration of sex work tended to be lower and the number of clients on the previous working day tended to be higher among ESN FSWs with a positive ELISPOT result, although these differences were not statistically significant. ESN FSWs with a positive result by IFN-γ secretion flow cytometry had significantly more clients on the previous working day and significantly higher numbers of exposures to HIV per month, compared with ESN FSWs with a negative result. ESN FSWs with a positive result tended to have a shorter duration of sex work and an increased proportion of unprotected sex. Interestingly, the duration of sex work among ESN FSWs was inversely associated with the number of clients on the previous working day ($r = -0.481$; $P = .002$) and directly with the proportion of unprotected sex ($r = 0.433$; $P = .005$) (figure 3). No association was found between the duration of sex work and the number of exposures to HIV per month ($r = 0.027$; $P = .867$).

DISCUSSION

The presence of HIV-specific T helper cells was assessed as a potential correlate of exposure to HIV in a population of African ESN FSWs. Low-level Gag- or Env-specific T helper responses were detected in 20% of ESN FSWs by use of the IFN-γ ELISPOT assay and in 38% of ESN FSWs by use of IFN-γ secretion flow cytometry. The detection of HIV-specific T helper responses was associated with the frequency, rather than with the duration, of exposure to HIV among ESN FSWs. These data indicate that HIV-specific T helper cells are primed in ESN FSWs but that their persistence depends on frequent and continuous exposure to HIV.

Despite intensive prevention efforts and increasing condom use, ESN FSWs in Abidjan are still frequently exposed to HIV and are at high risk of acquiring infection [23]. Indeed, ESN FSWs enrolled in this study were estimated to have a median of 4.3 unprotected exposures to HIV per month (or 52 exposures/year), which is close to the ⩾60 exposures to HIV per year reported for ESN FSWs in Nairobi [3]. ESN FSWs with a detectable HIV-specific T helper response had significantly more clients on the previous working day and more exposures to HIV per month and tended to have a lower total duration of commercial sex work. In concert with this finding, an inverse correlation between the total duration of commercial sex work and the number of clients on the previous working day was noted. Although we cannot exclude potential bias resulting from the self-reporting of sexual behavior, the extrapolation of these data, and the small number of subjects with detectable HIV-specific responses, these observations make sense in the light of previous reports. For example, among ESN FSWs in Nairobi, waning of CTL responses was detected in association with a break in sex work or a reduction in the number of clients per day [12]; HIV-specific responses in health care workers with occupational exposure to HIV rapidly decreased to below the limits of detection [17, 30]; reduced HIV-stimulated T helper cell activity was found in cord blood after short-course antiretroviral treatment for prevention of maternal-infant transmission [31]; the highest urethral concentrations of HIV-1–specific IgA were seen in ESN men with the most recent unprotected sexual episode [32]; and the majority of ESN women with HIV-specific T cell responses reported recent exposure to HIV [33]. Together, these findings may suggest that frequent and continuous exposure to HIV is the prerequisite for the presence and maintenance of HIV-specific immunity in ESN subjects.

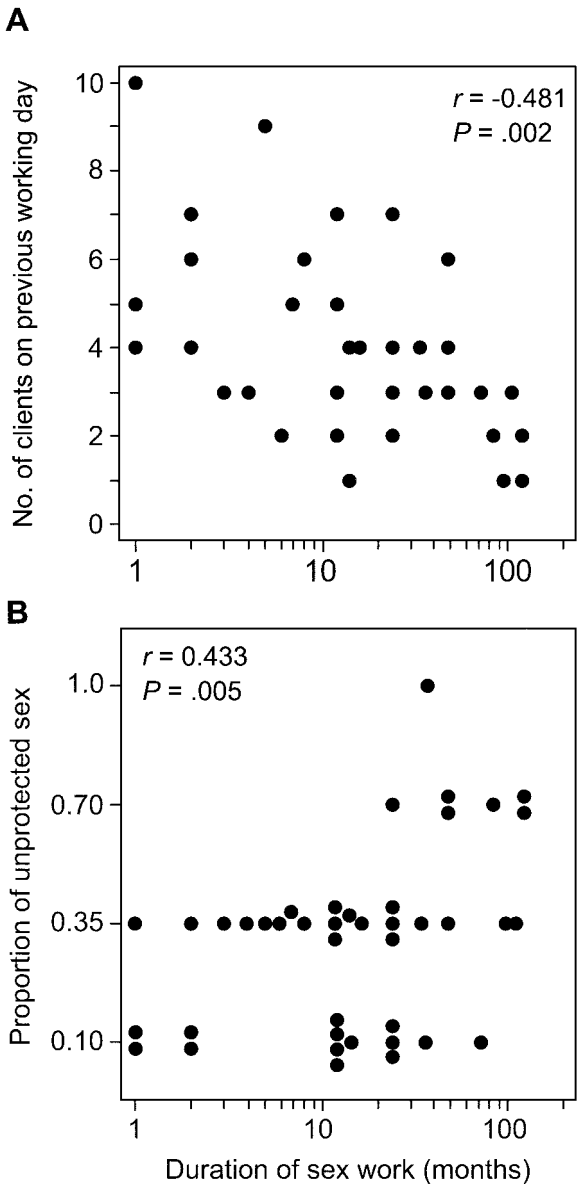

Figure 3. Sexual behavior characteristics of 40 HIV-exposed seronegative (ESN) female sex workers (FSWs). *A,* Correlation between the duration of commercial sex work and the no. of clients on the previous working day. *B,* Correlation between the duration of commercial sex work and the proportion of unprotected sex. The proportion of unprotected sex was based arbitrarily on the reported consistency in using condoms and was scored as follows: always, 0.1; often, 0.35; rarely, 0.7; and never, 1. Correlation analyses were performed with Spearman's rank correlation test.

In contrast to many other studies [5, 8, 33], including the present one, some studies have failed to detect HIV-specific T cell responses in ESN subjects [34, 35]. First, the absence of detectable HIV-specific T cells may have resulted from the applied methodology, including the use of preset negative cutoff values, which leads to decreased sensitivity of the ELISPOT assay (discussed below). In addition, the rate of exposure to HIV among HIV-1–discordant couples and other populations of ESN subjects is likely many-fold lower and less continuous than that among African FSWs [5, 35], so that the frequency of preexisting HIV-specific T cells in these subjects may have decreased below the limit of detection by the time of blood sampling. Finally, it is conceivable that other immune responses, such as HIV-specific IgA [21, 36] or noncytolytic $CD8^+$ T cell–mediated HIV inhibition [37, 38], contribute to protection against infection with HIV in these populations.

Detectable ELISPOT responses to HIV-1 proteins in our group of ESN FSWs were relatively weak (7–20 sfcs/10^6 PBMCs). Instead of using a preset cutoff value of 20 or 50 sfcs/10^6 PBMCs, as in other studies [8, 28], HIV protein ELISPOT responses in the present study were considered to be positive if they exceeded the average response plus 3 times the SD in a large group of HIV-negative female blood donors at lower risk of HIV infection, statistically limiting 99.7% of negative ELISPOT responses. Because assay conditions—such as the origin and quality of the samples, operators, and composition of the media—may vary for every study, this approach may be more justifiable than using preset cutoff values. In the present study, high ELISPOT sensitivity was obtained as a consequence of low background responses among all study subjects and very low HIV protein responses among HIV-seronegative female blood donors. The use of freshly isolated PBMCs, rather than cryopreserved PBMCs, and human serum, instead of fetal calf serum, in the culture medium could have been instrumental, together with the fact that the p24 and gp120 proteins selected for this study showed significantly lower background stimulation in low-risk HIV-seronegative laboratory workers, compared with a set of HIV proteins from other sources (authors' unpublished data). Thus, although of low level, HIV-specific T helper responses among ESN FSWs were within the detectable range of this sensitive ELISPOT assay. Moreover, experiments are being conducted with a highly sensitive modified ELISPOT method to confirm the presence of HIV-specific T helper cells in ESN FSWs [39].

The question of whether such low-level HIV-specific responses are of significance with respect to protection against infection with HIV remains unanswered. In that regard, the in vitro responses to HIV proteins observed in a subgroup of ESN FSWs in the present study may also be coincidental markers of increased exposure to HIV. Alternatively, they may have resulted from infection with cross-reactive pathogens, as recently proposed elsewhere [40, 41], or from exposure to cross-reactive alloantigens [42, 43], both of which may be experienced more often by ESN FSWs than by female blood donors. In fact, the protective role of T helper responses in ESN subjects can only be addressed in a longitudinal seroconversion study, for which, unfortunately, the present study was not designed.

ELISPOT and IFN-γ secretion flow cytometry assays had concordant results for the detection of HIV-specific responses

in HIV-seropositive subjects and for the detection of CMV-specific responses in all study subjects. Among ESN FSWs, however, the detection of HIV-specific T cells by IFN-γ secretion flow cytometry did not correlate with the results of the ELISPOT assay, which may have been the consequence of the low-level responses in these subjects, which were close to the limit of detection for both assays. In addition, discrepancies were noted in the magnitude of the responses detected by the 2 methods. Indeed, IFN-γ secretion flow cytometry may magnify responses, because IFN-γ secreted by HIV-specific T cells may be taken up by nonstimulated T cells in the vicinity, as suggested elsewhere [44].

Typically, exogenous viral proteins are taken up by antigen-presenting cells, processed, and presented on major histocompatibility complex (MHC) class II molecules to stimulate $CD4^+$ T cells. In the present study, however, some ESN and HIV-seropositive FSWs also had HIV-1 protein–stimulated $CD8^+$ T cells, in addition to $CD4^+$ T cells. This may have been the result of an alternate pathway for exogenous antigen processing and presentation on MHC class I molecules to $CD8^+$ T cells, referred to as "antigen cross-presentation" [45, 46]. The occurrence of this pathway in ESN FSWs in vivo could indicate that HIV-specific CTLs are not necessarily the result of an extraordinary controlled HIV infection in these subjects, as recently proposed elsewhere [47], but that $CD8^+$ T cells may be primed directly with defective nonreplicating particles or viral proteins. Of interest, antigen cross-presentation has been suggested to play a role in protection against HIV disease progression [48, 49], and it should therefore be studied more in ESN subjects as well.

Among HIV-seropositive FSWs, T helper responses against Gag p24 were much higher than responses against Env gp120. This may be explained by the fact that HIV-1 Gag is more conserved than HIV-1 Env in such a way that the clade B Gag p24 protein used in our analyses has more immunodominant epitopes in common with the circulating HIV-1 clade A/G viruses in Côte d'Ivoire than the clade B Env gp120 protein. Of interest, these differences between Gag and Env responses were not present among ESN FSWs, suggesting that ESN FSWs recognize as many conserved clade B epitopes in Env as they do in Gag. This result may be in line with the previous observation of a differential HIV-1 epitope recognition in ESN and HIV-seropositive subjects [11]. In that respect, it can be hypothesized that ESN FSWs in the present study responded to the rare conserved epitopes within the highly variable Env epitope, which may be more protective against HIV, whereas HIV-seropositive subjects responded to newly emerging but less protective Env epitopes.

In conclusion, low levels of HIV-specific T helper cells detected in a subgroup of ESN FSWs in Abidjan were associated with the frequency of exposure to HIV, rather than with the duration of sex work. These data may have important implications for HIV vaccine design and monitoring of immune responses in vaccine recipients.

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