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HIV-1-Specific Enzyme-Linked Immunosorbent Spot Assay Responses in HIV-1-Exposed Uninfected Partners in Discordant Relationships Compared to Those in Low-Risk Controls

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A number of studies of highly exposed HIV-1-seronegative individuals (HESN) have found HIV-1-specific cellular responses. However, there is limited evidence that responses prevent infection or are linked to HIV-1 exposure. Peripheral blood mononuclear cells (PBMC) were isolated from HESN in HIV-1-discordant relationships and low-risk controls in Nairobi, Kenya. HIV-1-specific responses were detected using gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assays stimulated by peptide pools spanning the subtype A HIV-1 genome. The HIV-1 incidence in this HESN cohort was 1.5 per 100 person years. Positive ELISpot responses were found in 34 (10%) of 331 HESN and 14 (13%) of 107 low-risk controls (odds ratio [OR] = 0.76; $P = 0.476$). The median immunodominant response was 18.9 spot-forming units (SFU)/10^6 peripheral blood mononuclear cells (PBMC). Among HESN, increasing age (OR = 1.24 per 5 years; $P = 0.026$) and longer cohabitation with the HIV-1-infected partner (OR = 5.88 per 5 years; $P = 0.003$) were associated with responses. These factors were not associated with responses in controls. Other exposure indicators, including the partner's HIV-1 load (OR = 0.99 per $\log_{10}$ copy/ml; $P = 0.974$) and CD4 count (OR = 1.09 per 100 cells/μl; $P = 0.238$), were not associated with responses in HESN. HIV-1-specific cellular responses may be less relevant to resistance to infection among HESN who are using risk reduction strategies that decrease their direct viral exposure.

Conventional vaccines exploit natural immune responses that occur upon exposure to pathogens and confer protection. Several groups have demonstrated that a subset of HIV-1-exposed adults and infants have evidence of HIV-1-specific cellular activity yet remain HIV-1 seronegative (2, 4, 5, 9, 14, 19, 22–27, 32, 36, 39, 40, 43–46, 48). However, there is limited evidence that these immune responses prevent HIV-1 infection, and controversy exists about their detection and linkage to HIV-1 exposure.

Non-HIV-1-infected partners in HIV-1-discordant relationships represent one of the largest populations of highly HIV-1-exposed seronegative individuals (HESN) in sub-Saharan Africa, where more than 50% of all couples affected by HIV-1 are discordant (30, 50). There are conflicting data on the detection and prevalence of HIV-1-specific $CD8^+$ T cell responses in this population; during the last decade, a number of studies in the United States, Europe, and Africa reported positive HIV-1-specific T cell responses at rates ranging from 10 to 75% among HESN in long-term relationships (2, 4, 5, 9, 14, 19, 22–27, 32, 36, 39, 40, 43–46, 48). In contrast, a recent study found no positive responses in an HIV-1-discordant couple cohort in Zambia after rigorous investigation (1). Many have suggested that discrepant results are due to cohort differences, especially variations in factors related to the level and timing of HIV-1 exposure (51), while others attribute them to differences in assay technique and sensitivity (7, 38, 49).

In this study, we hypothesized that examining factors associated with positive HIV-1-specific ELISpot responses could explain differences in results between cohorts, particularly when these factors are compared to a control population at low risk of exposure to HIV-1. Such analyses have not been routinely performed due to the large sample size required to examine correlates of immunity and the lack of a control population from the same region. In areas of high HIV prevalence, identifying an appropriate control population with characteristics similar to those of the study population can be challenging, because partner HIV-1 status is not always known or accurately reported and self-reporting of sexual activity may not be reliable. To address these limitations and to improve understanding of correlates of protective immune responses, we conducted a longitudinal cohort study of HIV-1-discordant couples and HIV-1-seronegative individuals in known HIV-1-negative relationships to investigate correlates of gamma interferon (IFN-γ) secretion following stimulation with HIV-1 peptide pools. HIV-1-specifc ELISpot assays were conducted in both HIV-1-discordant and -concordant HIV-1-negative couples, and so-

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ciodemographic, sexual behavior, medical history, and clinical data were collected from both partners in the couples.

MATERIALS AND METHODS

Study participants. HIV-1-discordant couples were recruited from voluntary counseling and testing (VCT) centers in Nairobi, Kenya, between September 2007 and May 2009. Eligible couples reported sex ≥3 times in the 3 months prior to screening, were not pregnant, and planned to remain together for the duration of the study. At enrollment, HIV-1-infected participants did not have a history of clinical AIDS (WHO stage IV) and were not currently on antiretroviral therapy (ART). Eligibility screening and couple counseling, including risk reduction and condom counseling, preceded the enrollment visit.

During the same period, couples in which both partners tested HIV-1 seronegative were recruited from the same VCT centers as the discordant couples. Concordant negative couples also had to report having sex with their partner ≥3 times in the 3 months prior to screening and were not pregnant at enrollment. Couples were ineligible if either partner reported sexual relationships outside the primary partnership.

Written informed consent was obtained from all participants. The study received ethical approval from the institutional review boards of the University of Washington and the University of Nairobi and was conducted according to the guidelines set forth by the U.S. Department of Health and Human Services.

Clinical procedures. At enrollment, clinical staff examined the participants, administered questionnaires to collect sociodemographic and sexual behavior data, and took a detailed medical history. Questions were presented in English or Kiswahili depending on participant preference, and interviews were conducted individually to ensure confidentiality. A self-reported number of sex acts with the study partner was recorded for both partners of the couple, and the mean number of acts reported by the couple was used for analysis. Urine pregnancy tests (Quick Vue One Step hCG Urine Pregnancy Kit; Quidel Corp., San Diego, CA) were done at enrollment and at each quarterly follow-up visit.

HIV-1, HSV-2, and syphilis diagnosis. Participants were tested for HIV-1 at enrollment and quarterly by two rapid tests conducted in parallel using a Determine HIV-1/2 rapid test (Abbott Laboratories, Tokyo, Japan; now marketed by Inverness Medical as Alere Determine) and the Bioline HIV 1/2 rapid test (Standard Diagnostics Inc., Suwon, South Korea) and were eligible only if they had concordant rapid test results at screening. Plasma from HIV-1-infected partners collected at enrollment was assayed for HIV-1 RNA load using Gen-Probe Transcription Mediated Amplification (Gen-Probe, San Diego, CA). This assay detects the prevalent HIV-1 subtypes in Kenya, subtypes A, C, and D (10, 11). Serum samples were also collected at enrollment from both partners to test for herpes simplex virus 2 (HSV-2) and syphilis. Testing for HSV-2 was performed using Focus enzyme-linked immunosorbent assay (ELISA) kits (Cyprus, CA) with a cutoff of 3.5 (15), and testing for syphilis used a rapid plasma reagin (RPR) test (Becton, Dickinson and Company, Franklin Lakes, NJ) with confirmation by *Treponema pallidum* hemagglutination assay (TPHA) (Randox Laboratories, Crumlin, United Kingdom).

IFN-γ ELISpot assays. IFN-γ ELISpot assays were used to assess cellular responses in partners in HIV-1-discordant relationships and in individuals in concordant HIV-1-seronegative partnerships. EDTA-anticoagulated blood samples were collected at enrollment, and peripheral blood mononuclear cells (PBMC) were isolated using density gradient separation. HIV-1-specific cellular responses were assessed using an IFN-γ ELISpot assay, as described previously, with modifications (28, 31). Briefly, 96-well MAIP45 nitrocellulose plates (Millipore Corporation, Billerica, MA) were coated with monoclonal antibody to IFN-γ (Mabtech, Nacka, Sweden) and blocked with RPMI 1640 supplemented with 10% fetal calf serum and 20 mM L-glutamine (R10) (all Sigma) before adding 1×10^5 PBMC to each well. Triplicate experimental wells were stimulated with pools of HIV-1 subtype A peptides comprised of 15-mers overlapping by 10 amino acids spanning the HIV-1 genome and reconstituted according to the manufacturer's instructions (PEPscreen; Sigma-Aldrich Corporation, St. Louis, MO). Twenty-two peptide pools were generated containing ~40 sequential peptides per pool. Cells in 3 positive-control wells were stimulated with phytohemagglutinin (PHA) (Murex, Biotech Ltd., Dartford, United Kingdom), and cells in 9 negative-control wells were incubated with R10 alone. After overnight incubation, the cells were removed from the plates by washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, followed in sequence by application of biotinylated anti-IFN-γ antibody, washing, and streptavidin-conjugated alkaline phosphatase (Mabtech). After the final washing, spot-forming units (SFU) were visualized by the addition of alkaline phosphatase substrate (Mabtech). The plates were dried overnight before being counted and quality controlled using ImmunoSpot software on a CTL ImmunoSpot S4 Core Analyzer (Cellular Technology Ltd., Shaker Heights, OH).

Statistical methods. The mean number of SFU/10^6 PBMC across the triplicate stimulated wells was determined for each of the 22 peptide pools. The mean background was calculated using the 9 unstimulated negative-control wells. The number of peptide-specific SFU/10^6 PBMC was calculated by subtracting the mean background from the mean crude SFU/10^6 PBMC from the stimulated wells. Positive IFN-γ responses were defined as a mean of ≥50 peptide-specific SFU/10^6 PBMC and more than twice the mean SFU/10^6 PBMC across the negative-control wells. Participants were excluded from analysis if positive-control wells were <50 SFU/10^6 PBMC or if there was no difference in mean SFU/10^6 PBMC in positive- and negative-control wells. A participant was defined as a responder if he/she had ≥1 peptide pool with a positive response. The final analysis was restricted to those with low background response, defined as a mean of <50 SFU/10^6 PBMC across unstimulated negative-control wells. Sociodemographic, behavioral, and biological characteristics of the couple and the individuals within the couple were assessed as correlates of the presence of HIV-1-specific T cell IFN-γ responses. For univariate comparisons, odds ratios (OR), 95% confidence intervals (CI), and *P* values were calculated based on Fisher's exact test. Logistic regression was used for continuous variables. The partner's plasma viral load was $\log_{10}$ transformed, and all analyses were conducted using Stata statistical software (Stata Corp., College Station, TX).

RESULTS

Participant characteristics. Among 408 HIV-1-discordant couples, there were 144 (35%) female HESN and 264 (65%) male HESN. The median age of HESN was 32 (interquartile range [IQR], 27 to 38) years, the median age at sexual debut was 18 (IQR, 16 to 20) years, and the median number of lifetime sexual partners was 4 (IQR, 2 to 6). Participants reported a median of 5 (IQR, 2.5 to 8) sex acts with their HIV-1-infected partner in the past month, and 100 (25%) reported unprotected sex in the past month. The median length of cohabitation was 5.0 (IQR, 2.0 to 10.0) years. Over the period of study follow-up, a total of 58 (14%) HIV-1-discordant couples experienced at least 1 pregnancy, and in 12 (3%) couples, the initially non-HIV-1-infected partner seroconverted. The seroconversion rate was 1.5 per 100 person years.

There were some relevant differences between male and female participants (Table 1). A larger proportion of males reported a history of sexually transmitted infection (STI) (42% versus 21%), and a smaller proportion were HSV-2 seropositive compared to female participants (41% versus 68%). Couples with a male non-HIV-1-infected partner reported slightly more sex acts in the past month (5.5 versus 4.0), and these couples tended to have an infected partner with higher CD4 counts (456.0 versus 359.0 cells/μl) and lower plasma RNA viral loads (4.6 versus 4.8 $\log_{10}$ RNA copies/ml).

Sixty-four HIV-1-seronegative females and 65 seronegative males in monogamous, concordant HIV-1-negative relationships

TABLE 1 Baseline characteristics of HIV-1-infected partners in HIV-1-discordant couples enrolled in the prospective cohort study compared to low-risk controls, by gender

Characteristic	Median (IQR) or *n* (%)			
	HESN		Controls	
	Male (*n* = 264)	Female (*n* = 144)	Male (*n* = 65)	Female (*n* = 64)
Age (yr)	34 (29, 40)	29 (25, 35)	30 (26, 33)	25 (22, 30)
Less than primary education (*n*)	40 (15.2)	38 (26.4)	6 (9.2)	9 (14.1)
Age at 1st sex (yr)	18 (16, 20)	18 (16, 20)	17 (15, 19)	18 (16, 20)
Lifetime sexual partners (*n*)	5 (3, 9)	3 (2, 3)	5 (34, 9)	3 (2, 3)
Yr living together	4.0 (2.0, 9.0)	5.3 (2.0, 10.5)	2.0 (0.0, 7.0)	2.0 (0.0, 7.0)
Sex acts (*n*)[a]	5.5 (3, 9)	4 (2, 8)	4.0 (2, 9)	4.0 (2.0, 12.0)
Any unprotected sex (*n*)[a]	68 (25.8)	32 (22.2)	50 (76.9)	46 (71.9)
Married (*n*)	252 (95.5)	140 (97.2)	40 (61.5)	42 (66.6)
Desire future children (*n*)	134 (51.0)	59 (41.3)	48 (73.8)	50 (78.1)
History of STI (*n*)	106 (41.6)	29 (20.9)	22 (33.8)	15 (24.2)
Malaria (*n*)[b]	19 (7.2)	14 (9.7)	4 (6.2)	6 (9.4)
Genital sores (*n*)[b]	17 (6.4)	9 (6.3)	1 (1.5)	2 (3.1)
Circumcised (*n*)	223 (84.5)		57 (87.7)	
HSV-2 seropositive (*n*)	109 (41.3)	98 (68.1)	15 (23.8)	19 (31.7)
Syphilis (*n*)	4 (1.5)	1 (0.7)	0 (0.0)	0 (0.0)
BV (*n*)[c]		23 (16.1)		14 (29.8)
Cervicitis (*n*)		1 (0.7)		0 (0.0)
Vaginitis (*n*)		8 (5.6)		3 (6.3)
Partner's CD4 count (no. of cells/μl)	456.0 (303.0, 638.0)	359.0 (254.0, 533.0)		
Partner's plasma viral load (no. of $\log_{10}$ RNA copies/ml)	4.6 (3.8, 5.2)	4.8 (4.1, 5.4)		

[a] With study partner in the past month.
[b] Self-reported in the past 3 months.
[c] BV, bacterial vaginosis.

were included as low-risk negative controls, and 27 HIV-1-infected partners in discordant relationships (26% male; 74% female) were included as positive controls. Low-risk controls were generally comparable to HESN, but the low-risk controls were younger (median age, 27 versus 32 years), reported a shorter time living together (median, 2.0 versus 5.0 years), and reported more unprotected sex in the past month (74% versus 25%). Compared to HESN, a smaller proportion of low-risk controls reported less than a primary education (12% versus 19%), were married (64% versus 96%), and were HSV-2 seropositive (28% versus 51%). A larger proportion of low-risk controls reported a desire for additional children (76% versus 48%).

As described elsewhere, the HIV-1-infected partners in the discordant-couple relationships were generally similar in demographic and behavioral characteristics to the uninfected partners (16, 17).

Prevalence and factors associated with positive ELISpot responses. Sixty-two (15%) HESN and 23 (18%) low-risk controls had positive IFN-γ ELISpot responses detected following stimulation with HIV-1 peptides (OR = 0.83; 95% CI, 0.48 to 1.47; P = 0.49). When restricted to those with low background response (mean, <50 SFU/10^6 PBMC), positive responses were identified in 34 (10%) of 331 HESN and 14 (13%) of 107 negative controls (OR = 0.76; 95% CI, 0.38 to 1.60; P = 0.48). All subsequent analyses were restricted to those with low background response.

The maximum, or immunodominant, IFN-γ response was determined for each participant by pool. The median immunodominant pool response among HESN was 18.9 peptide-specific SFU/10^6 PBMC (IQR, 11.1 to 32.2 SFU/10^6 PBMC), and for low-risk controls it was 23.3 peptide-specific SFU/10^6 PBMC (IQR, 15.6 to 34.4 SFU/10^6 PBMC) (P = 0.015). Among HIV-1-infected partners assessed as positive controls, we found 23 (95.8%) of 24 had a positive ELISpot response. The median immunodominant pool response among HIV-1-infected positive controls was 513.3 SFU/10^6 PBMC (IQR, 211.7 to 1,010.6 SFU/10^6 PBMC).

The relative distributions of responses by viral region were similar for HESN and low-risk controls, with the largest proportion of responses to accessory proteins (tat, rev, vif, vpu, and vpr) (68% of HESN responders and 57% of low-risk control responders), followed by pol (38% of HESN responders and 50% of low-risk control responders), and the smallest proportion of responses were against gag (26% of HESN responders and 14% of low-risk control responders) and nef (24% of HESN responders and 14% of low-risk control responders) (Fig. 1a). Although not significantly different from low-risk controls, HESN responded in higher proportions to gag, nef, env, and accessory proteins and in smaller proportions to pol. Among responders, the largest proportion of both HESN (53%) and low-risk controls (79%) responded to just 1 viral region, with a trend indicating that HESN may have been more likely to respond to 2 or more viral regions than low-risk controls (OR = 3.26; 95% CI, 0.67 to 20.94; P = 0.12) (Fig. 1b). In comparison, HIV-1-infected positive controls differed qualitatively from HESN and low-risk controls in the viral regions to which they responded. Approximately equal proportions of the 23 HIV-1-infected controls with a positive ELISpot response recognized gag (70%), nef (61%), pol (74%), and accessory proteins (74%), and a smaller proportion (26%) responded to env. Among HIV-1-infected controls with positive responses, nearly all (91%) responded to ≥2 viral regions and 43% responded to ≥4 viral regions.

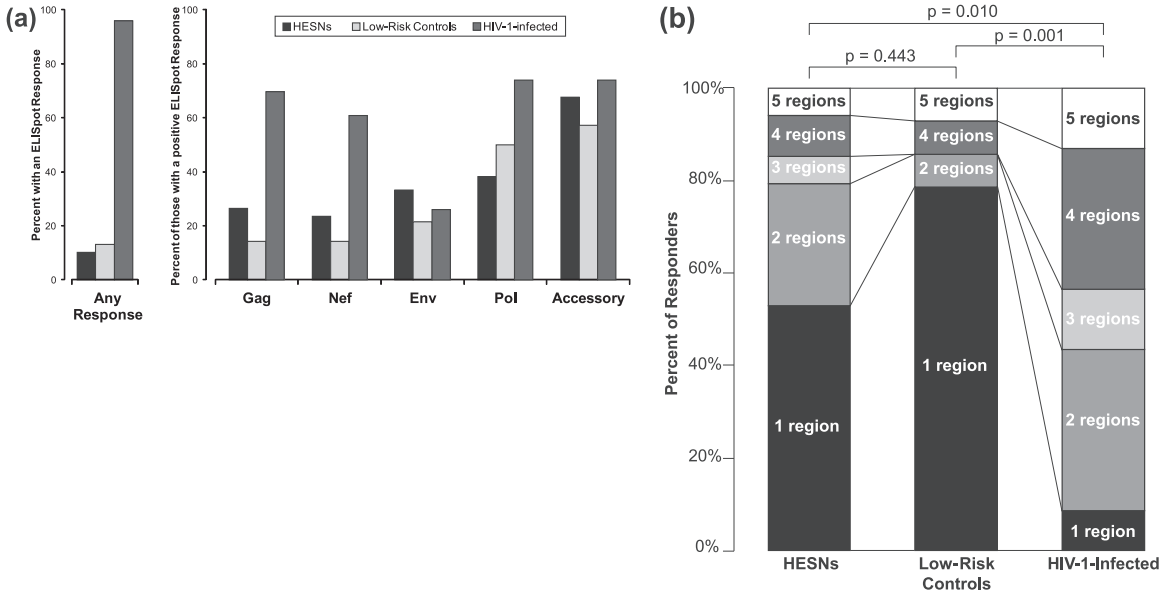

FIG 1 IFN-γ ELISpot assays were conducted on samples from HESN from HIV-1-discordant couples and low-risk controls from concordant HIV-negative couples using 1×10^5 cultured PBMC with 3 wells per peptide pool. Pools with ≥50 HIV SFU/10^6 PBMC and >2 times the background were defined as positive responses. A response to any peptide pool covering the viral region was counted as a region response (restricted to samples with a background of <50 SFU/10^6 PBMC). (a) T cell IFN-γ ELISpot responses by viral region. (b) Breadth of responses based on the number of viral regions to which participants mounted a response. The *P* values are for χ^2 tests comparing the distributions between groups.

Correlates of positive ELISpot responses. Among HESN, we observed that increasing age, longer time living with the HIV-1-infected partner, and having less than a primary education were significantly associated with having a cellular response to HIV-1 peptides (Table 2). For every 5-year increase in age, there was a 1.24-fold (95% CI, 1.03 to 1.51; $P = 0.026$) increased likelihood of a response, and for every 5-year increase in time living with their study partner, there was a 5.88-fold (95% CI, 1.80 to 19.16; $P = 0.003$) increased likelihood of a response. Those with less than a primary education were 2.45-fold (95% CI, 1.04 to 5.54; $P = 0.024$) more likely to have a response than those with at least a primary education. However, in comparison, among low-risk control participants, point estimates for age (OR = 0.64; 95% CI, 0.38 to 1.06; $P = 0.083$), duration living with their study partner

TABLE 2 Correlates of ELISpot responses among HESN[a]

Characteristic	Median (IQR) or *n* (%)		OR[b]	95% CI	*P* value
	Responders (*n* = 34)	Non-responders (*n* = 297)			
Female (*n*)	10 (29.4)	106 (35.7)	0.75	0.31–1.71	0.571
Pregnancy during study (*n*)	3 (8.8)	42 (14.1)	0.59	0.11–2.02	0.596
Age (yr)	32.5 (30, 46)	32 (27, 38)	1.24[f]	1.03–1.51	0.026
Less than primary education (*n*)	12 (35.3)	54 (18.2)	2.45[f]	1.04–5.54	0.024
Yr living together	6.5 (2.0, 13.0)	4.0 (2.0, 9.0)	5.88[g]	1.80–19.16	0.003
Lifetime partners (*n*)	4.0 (3.0, 5.0)	4.0 (2.0, 6.0)	0.97	0.90–1.04	0.423
Sex acts (*n*)[c]	5.3 (2.0, 8.0)	5.0 (2.5, 8.5)	0.95	0.91–1.07	0.788
Unprotected sex (*n*)[c]	10 (29.4)	82 (27.6)	1.09	0.45–2.50	0.841
History of STI (*n*)	11 (33.3)	105 (36.1)	0.89	0.37–2.00	0.849
Malaria (*n*)[d]	2 (5.9)	27 (9.1)	0.63	0.07–2.70	0.752
Genital sores (*n*)[d]	0 (0.0)	24 (8.1)	0	0–1.30	0.152
HSV-2 seropositive (*n*)	17 (50.0)	149 (50.2)	0.99	0.46–2.16	0.999
Circumcised (*n*)[e]	22 (91.7)	166 (86.9)	1.66	0.37–15.37	0.746
Partner's CD4 count (no. of cells/μl)	476.0 (303.0, 617.0)	400.5 (278.0, 617.0)	1.09	0.94–1.26	0.238
Partner's plasma viral load (no. of $\log_{10}$ RNA copies/ml)	4.8 (3.9, 5.4)	4.7 (4.0, 5.3)	1.00	0.74–1.34	0.974

[a] Analysis is restricted to samples with background of <50 SFU/10^6 PBMC. Odds ratios.
[b] The OR for age and years living together is per 5 years. The OR for partner's CD4 count is per 100 cells/μl, and the OR for partner's plasma viral load is per $\log_{10}$ RNA copies/ml.
[c] With study partner in the past month.
[d] Self-reported in the past 3 months.
[e] Males only.
[f] $P < 0.05$.
[g] $P < 0.001$.

TABLE 3 Correlates of ELISpot responses among low-risk controls[a]

Characteristic	Median (IQR) or *n* (%)		OR[b]	95% CI	*P* value
	Responders (*n* = 14)	Nonresponders (*n* = 93)			
Female (*n*)	11 (78.6)	42 (45.2)	4.45[f]	1.07–26.12	0.023
Age (yr)	23.5 (21, 27)	28 (25, 33)	0.64	0.38–1.06	0.083
Less than primary education (*n*)	1 (7.1)	9 (9.7)	0.72	0.02–6.02	0.999
Yr living together	0.8 (0.0, 5.0)	2.4 (0.0, 8.0)	0.70	0.38–1.28	0.246
Lifetime partners (*n*)	3.0 (2.0, 5.0)	3.0 (3.0, 6.0)	0.98	0.91–1.05	0.533
Sex acts (*n*)[c]	8.0 (4.0, 9.0)	4.0 (2.0, 12.0)	0.99	0.92–1.08	0.978
Unprotected sex (*n*)[c]	13 (92.9)	65 (69.9)	5.60	0.76–246.31	0.106
History of STI (*n*)	7 (50.0)	23 (25.3)	2.96	0.78–10.95	0.108
Malaria (*n*)[d]	1 (7.1)	6 (6.5)	1.12	0.02–10.42	0.999
Genital sores (*n*)[d]	0 (0.0)	2 (2.2)	0	0–13.40	0.999
HSV-2 seropositive (*n*)	4 (30.8)	23 (25.6)	1.29	0.27–5.20	0.739
Circumcised (*n*)[e]	3 (100.0)	44 (86.3)	NA	0.11–∞	0.999

[a] Analysis is restricted to samples with background of <50 SFU/10^6 PBMC.
[b] OR for age and years living together are per 5 years. NA, not applicable.
[c] With study partner in the past month.
[d] Self-reported in the past 3 months.
[e] Males only.
[f] $P < 0.05$.

(OR = 0.70; 95% CI, 0.38 to 1.28; $P = 0.246$), and low education (OR = 0.72; 95% CI, 0.02 to 6.02; $P = 0.99$) were not significant and showed a direction of association opposite that observed for HESN (Table 3).

There was no significant association between IFN-γ responses and the infected partner's plasma viral load (OR = 0.99; 95% CI, 0.74 to 1.34; $P = 0.974$) or CD4 count (OR = 1.09 per 100 cells/μl; 95% CI, 0.94 to 1.26; $P = 0.238$). There were also no associations with HESN gender, number of reported sex acts, unprotected sex, HSV-2 serostatus, reports of genital ulcers in either partner, or male circumcision status. Among the low-risk controls, females were significantly more likely to have a positive response (OR = 4.45; 95% CI, 1.07 to 26.12; $P = 0.042$), but for all other potential correlates of HIV-1 exposure, there were no significant associations with positive ELISpot responses.

DISCUSSION

This is one of the largest studies of immune responses among HESN, with more than 400 *ex vivo* assays performed on samples collected from men and women in HIV-1-discordant couples. This study also presents data from an exceptionally large control population comprised of 127 HIV-1-negative individuals in monogamous relationships. Among HESN, 10% had positive IFN-γ ELISpot responses using standard criteria, and these responses were associated with older age and increased relationship duration. This is similar to what was found by Kaul and colleagues among commercial sex workers in Kenya: longer duration of sex work was associated with a positive response, and women who continued to engage in sex work without becoming infected were more likely to have responses than their younger counterparts (24). Such predictors are consistent with the hypothesis that ELISpot responses are more than a marker of exposure and may be part of a protective immune response, so that those individuals who do not seroconvert over time despite ongoing exposure are the ones most likely to have responses. While the failure of candidate vaccines that successfully elicit robust HIV-1-specific IFN-γ responses demonstrates that an IFN-γ response alone is not sufficient for protection (6, 34), there remains evidence that such responses, in the context of natural exposure, are at least a good correlate of protection (19, 21).

In this study, our intention was to evaluate whether HIV-1-specific cellular responses were protective; however, this was not possible, because there were too few HIV transmission events among enrolled couples. As in any discordant-couple cohort, there is major emphasis on HIV-1 prevention counseling and provision of condoms to couples, resulting in reduced exposure. This cohort had a low incidence rate of HIV-1 transmission at 2.1 per 100 person years for male-to-female and 1.1 per 100 person years for female-to-male transmission. This was a lower transmission rate than has been described in many previous studies of discordant couples (18) but is similar to the rates found in recent large clinical trials involving ongoing prevention counseling (8). From this, we can deduce that there was relatively low HIV-1 exposure. We have no reason to suspect that HIV-1-exposed, uninfected partners used postexposure (PEP) or preexposure (PrEP) prophylaxis that would have resulted in fewer HIV-1 infections despite high exposure. Questions regarding antiretroviral use were asked during exit interviews (data not shown), and this possibility has been assessed in similar cohorts in Nairobi with negative results. Low exposure levels, likely due to high levels of condom use, may explain the lack of observed associations with other markers of exposure, such as the HIV-1 load in the infected partner, frequency of sex, and genital ulcer disease. Our findings in this HESN cohort using overlapping peptide pools contrast with a previous study by our group among 161 HIV-exposed, uninfected infants in which we found a positive association between exposure to HIV-1 and HIV-1-specific ELISpot responses using defined $CD8^+$ cytotoxic T lymphocyte (CTL) epitopes. We also found an association between infant ELISpot responses and protection against infection. Levels of exposure were likely to have been much greater in this previous cohort, which had a transmission rate of >15% overall (12, 19).

Another important observation in our study is that a relatively high proportion of low-risk controls had positive responses. Among 23 publications we reviewed, 20 assessed responses in a control population (although not always from the same popula-

TABLE 4 Prevalence of HIV-1-specific cellular responses among HESN and low-risk controls in previously published studies ordered from lowest to highest prevalence in each category

Study	HESN		Low-risk controls		Assay
	n	Prevalence (%)	*n*	Prevalence (%)	
Exposed, uninfected individuals					
Makedonas et al. (32)	47	28	19	0	Peptide CD8 IFN-γ ELISpot
Goh et al. (14)	36	36	15	7	Recombinant VV-CTL[a] precursor frequency
Bernard et al. (4)	17	41	14	0	Recombinant VV-CTL
Missale et al. (36)	3	67	NR[b]	0	Peptide CD8 IFN-γ Elispot and ICS[c]
Commercial sex workers					
Kaul et al. (25)	39	21	0		Recombinant VV-CTL
Rowland-Jones et al. (43)	9	33	14	0	Peptide CTL
Alimonti et al. (2)	13	39	0		Peptide CD8 IFN-γ ICS
Kaul et al. (24)	91	47	18	0	Peptide CD8 IFN-γ ELISpot
Rowland-Jones et al. (44)	21	48	6	0	Peptide CTL
Kaul et al. (23)	16	69	7	0	Peptide CD8 IFN-γ ELISpot
Discordant couples					
Addo et al. (1)	28	0	15	0	Peptide IFN-γ ICS
Ritchie et al. (42)	16	0	21	0	Peptide IFN-γ ELISpot
Perez et al. (39)	23	13	13	0	Peptide IFN-γ ELISpot and ICS
Kebba et al. (26)	28	20	12	0	Peptide IFN-γ ELISpot
Shacklett et al. (47)	8	38	5	0	Peptide IFN-γ ELISpot using dendritic cell presentation
Skurnick et al. (48)	17	41	20	NR	Recombinant VV-IFN-γ ELISpot
Bienzle et al. (5)	11	45	6	0	Peptide CTL
Promadej et al. (40)	18	50	12	0	Recombinant VV-IFN-γ ELISpot
Schenal et al. (45)	15	>50	8	12.5	Peptide IFN-γ ICS
Ritchie et al. (42)	24	53 to 61	27	26 to 44	Cultured IFN-γ ELISpot
Kebba et al. (27)	10	75	5	0	Antigen CD4 ICS
Infants born to HIV-1-infected mothers					
John-Stewart et al. (19)	217	12–22	20	0	Peptide CD8 IFN-γ ELISpot
Schramm et al. (46)	106	13	20	0	Peptide IL-2[d] production ELISA
Clerici et al. (9)	8	38	7	0	Peptide CD8 IFN-γ ELISpot

[a] VV-CTL, vaccinia virus CTL.
[b] NR, not reported.
[c] ICS, intracellular cytokine staining.
[d] IL-2, interleukin-2.

tion used to recruit HESN), with a mean sample size of 13 individuals. This low number of controls greatly limited their power to detect low-level responses (Table 4). We found that 13% of our low-risk controls had positive responses compared to 10% of HESN, which may indicate nonspecificity of the ELISpot assay. This finding highlights the importance of using control populations that are both large enough to detect low proportions of responses and drawn from the same source population as the subjects of interest. Despite comparable proportions of responders among HESN and low-risk controls, it is interesting that responses among the low-risk controls were not correlated with any of the factors found to be correlates of responses among HESN. This may be due to chance, but it may also represent differences between the two populations in terms of factors influencing the specificity of the assay. In particular, the low-risk controls were far more likely to report unprotected sex, which may increase the likelihood of a recent genital infection. This may result in differences in immunologic responses that make the ELISpot assay less specific and could explain the larger proportion of female low-risk controls with ELISpot responses. In a large cohort of discordant couples, ~30% of new infections were linked to external partnerships (37), and it is possible that the "low-risk" controls in our study in fact had other exposures to HIV-1. These findings emphasize the challenges of interpreting HIV-1-specific responses that occur at low levels in a small proportion of subjects.

The high rate of weak positive responses among both HESN and low-risk controls raises questions about the utility of the ELISpot assay for measuring low-level responses among HESN. Similar to other studies among HIV-1-positive individuals, we found strong responses among approximately 96% of HIV-infected individuals, and these were directed equally against HIV-1 gag, pol, nef, and accessory-protein epitopes (24, 41). One hypothesis is that the ELISpot assay, when used in this population, is highly sensitive but less specific, which could contribute to increased rates of false-positive results in settings where true-positive results are infrequent. As with any test, when the prevalence of the outcome is lower (which would occur as studies move from HIV-1-infected individuals to HESN to candidate HIV vaccine trials to low-risk controls), there are proportionally more false-positive results, and the positive predictive value is lower.

This study had several strengths, including the large cohort size, the presence of large low-risk and positive-control populations, performance of assays on fresh samples to optimize sensitivity, and collection of detailed clinical histories and interviews for both partners in the relationship. The study was limited by the fact that we performed only one type of immune assay on unfractionated PBMC, making it difficult to distinguish $CD8^+$ and $CD4^+$ responses. CD4 responses to peptides restricted to multiple HLA-DR types have recently been shown to contribute to ELISpot responses observed in HESN and low-risk controls (42). We had limited ability to confirm responses using frozen samples due to limited numbers of PBMC. Stimulating peptide pools spanned the subtype A genome, and it is possible that a lack of cross-subtype reactivity resulted in lower response rates among those exposed to non-A subtypes; however, clade A accounts for >70% of HIV-1 infections in Kenya, and there is strong evidence of cross-clade ELISpot reactivity (3, 13, 20, 29, 33, 35, 52, 53). Finally, assays were performed in an unblinded manner. Despite these limitations, we believe this study contributes substantially to the existing literature on ELISpot responses among HESN.

In conclusion, we found that HIV-1-specific ELISpot responses in HESN were associated with the duration of HIV-1 exposure but that these responses did not occur in HESN at a level above what was found in low-risk controls. HIV-1-specific cellular immune responses have been found consistently in HESN who have persistent high levels of viral exposure. However, our findings, combined with other recent reports, indicate that among some HESN, these responses are reduced and may not contribute substantially to resistance to infection. This may be partially due to risk reduction strategies that decrease direct viral exposure, leading to new questions about the impact of such strategies on HIV-specific immune responses in HESN. Since weak positives also occurred in a relatively small proportion of control subjects in our study, the ELISpot assay was not sufficiently specific to investigate these rare and low-magnitude responses. Nonetheless, because the ELISpot assay can be readily conducted in large volume in resource-limited settings, it is likely to continue to play an important role in defining immune responses to HIV-1 and other pathogens. In future studies, adequate numbers of control subjects from the same source population as the HESN would enhance the validity and interpretability of results.

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