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# HIV-Specific Cytotoxic T Lymphocytes, HLA-A11, and Chemokine-Related Factors May Act Synergistically to Determine HIV Resistance in CCR5 Δ32-Negative Female Sex Workers in Chiang Rai, Northern Thailand

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## ABSTRACT

**Understanding how highly HIV-exposed individuals remain HIV uninfected may be useful for HIV vaccine design and development of new HIV prevention strategies. To elucidate mechanisms associated with resistance to HIV infection, immunologic and genetic factors were examined in 14 HIV-exposed but persistently seronegative (HEPS) female sex workers from Chiang Rai, northern Thailand and in ethnically matched, HIV-positive ($n$ = 9) and HIV-negative women ($n$ = 9). The HEPS women were identified in a study of commercial sex workers who had an HIV-1 incidence of 20.3 per 100 person-years. A high frequency of HLA-A11 was observed in HEPS women (86%) compared with northern Thai controls (56%). HIV-specific cytotoxic T lymphocyte (CTL) lytic responses were detected in cryopreserved peripheral blood mononuclear cells (PBMCs), using HLA-A-matched subtype E HIV-1 peptides in four of seven (57%) HEPS women, eight of eight HIV-positive women, and zero of nine HIV-negative unexposed controls ($p$ = 0.019 HEPS women vs. HIV-negative controls). CTL lysis levels were low, but responses were detected to peptides from Nef, Pol, Gag, and Env. Nef responses predominated in HEPS women. Compared with controls, HEPS women tended to have higher frequencies of CCR5 promotor 59402GG and SDF-1 3′UTR 801A genotypes known to influence HIV transmission or course of disease. HEPS women also had higher levels of spontaneous RANTES production by PBMCs than other groups. Each of these factors could potentially contribute to HIV resistance. As most HEPS women had one or more of these factors, they may prevent HIV infection synergistically by blocking HIV cell entry, delaying its dissemination, or killing HIV-infected cells.**

## INTRODUCTION

IMPROVED UNDERSTANDING of the factors that determine why some highly HIV-exposed individuals remain uninfected may enable the development of new strategies to prevent HIV infection. In Thailand, an estimated 1 million HIV infections have occurred since the late 1980s, the beginning of the epidemic in that country.[1] Thus there is an urgent need for effective HIV prevention measures such as vaccines.

The likelihood of HIV infection depends on the route of exposure, the level of infectiousness, and the frequency of HIV exposure.[2–4] Highly HIV-exposed persons, such as sex workers, injection drug users, partners of HIV-positive persons, and children of HIV-infected women, have particularly high inci-

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dence rates. Characteristics of the host also play a role. For example, the lack of HIV-1 coreceptor CCR5, the presence of HIV-specific cytotoxic or helper T cell responses, particularly HLA alleles, mucosal HIV-specific IgA, and soluble HIV-suppressive factors, may prevent or reduce the risk of HIV infection, while coinfections with other sexually transmitted diseases (STDs) may increase this risk.[3,5–22] Rare subsets of highly exposed persons who resist HIV infection have been identified in a variety of settings.[6–19,22,23] These apparently HIV-resistant persons are HIV negative by all standard serologic and polymerase chain reaction (PCR) assays. Several nomenclatures and classifications have been used to identify such persons: highly exposed persistently seronegative (HEPS), exposed uninfected (EU), and exposed seronegative (ES). The term HEPS is used in this study, as the acronym encompasses high exposure and allows for the possibility of an atypical or compartmentalized HIV infection as a component of the phenotype.

Several explanations for this apparent resistance to HIV-1 infection have been proposed, including that the HEPS person(s) or their cells never become HIV infected, develop a transient infection that is cleared, or develop an atypical, compartmentalized, chronic infection. Available data from HEPS studies could support all three scenarios.

The ability of the host to resist HIV infection is likely to be the result of many factors. The unique genetic background of some individuals, together with their propensity toward certain immune or other antiviral responses, probably combine to determine whether they will resist HIV infection. We have characterized several factors that influence HIV transmission in 14 apparently HIV-resistant women from Chiang Rai, northern Thailand. These women were identified in a cohort of brothel-based female sex workers (FSWs) who had an HIV-1 incidence rate of 20 per 100 person-years in the first year of follow-up; almost all infections were with HIV-1 subtype E.[24] We examined multiple immunologic and genetic factors that may reduce HIV transmission. A unique feature of these studies is that HIV-specific T cell responses in HEPS individuals as well as HIV-positive or -negative, unexposed persons were tested under *in vitro* conditions optimized to match for factors that might affect these responses, such as HLA class I allele type.

## MATERIALS AND METHODS

### *Study population and sample collection*

From 1991 to 1994, 500 FSWs, including 280 brothel-based workers, were enrolled in a prospective cohort study designed to examine factors associated with HIV infection in Chiang Rai, northern Thailand. At enrollment, 32% of the women were HIV seropositive, including 47% of the brothel workers.[25] Women were scheduled for study visits every 3 months. The incidence of seroconversion in the first year of follow-up was 20.3 per 100 person-years among brothel FSWs.[24] HIV-1 subtype E accounted for most infections.[24–27] During 1994 to 1996, 14 FSWs who remained in active cohort follow-up were identified as being persistently HIV-1 seronegative (HEPS) on the basis of the following criteria: (1) a history of working in a brothel in northern Thailand for $\geq$3 years, (2) HIV seronegative on $\geq$3 study visits, (3) seroreactive on herpes simplex virus type 2 (HSV-2)-specific immunoblot testing, and (4) positive on a syphilis *Treponema pallidum* hemagglutination assay (TPHA).[25] As part of a study protocol approved by the Ethical Review Committee of the Thai Ministry of Public Health (Nonthaburi, Thailand) and the Institutional Review Board of the Centers for Disease Control and Prevention (CDC, Atlanta, GA), these HEPS women were evaluated with various assays and findings were compared with those from nine ethnically matched HIV-infected FSWs from the cohort and nine HIV-seronegative female health care workers from Chiang Rai thought to be HIV unexposed. The HIV-infected FSWs and the health care workers were selected as convenient samples, but were HLA-A locus matched to the HEPS women (see below). Serum, plasma, and peripheral blood mononuclear cells (PBMCs) were collected during 1994–1995, in 1996, and in 1998 as previously described.[28] Because all 14 HEPS FSWs were not available at each time point and because only limited sample volumes were obtained, not all genetic or HIV DNA studies could be performed in each of the 14 HEPS women (below). All cellular immunology assays were performed on cryopreserved PBMCs. Three previously studied HIV-positive FSWs from the Chiang Rai cohort were also included for CCR2 and RANTES (regulation on activation normal T cell expressed and secreted) genetic studies.[28]

### *HIV and STD testing*

Serum or plasma specimens were tested for HIV-1 and HIV-2 antibodies with an HIV-1/HIV-2 enzyme immunoassay (EIA) (Genetic System, Redmond, WA). EIA-positive specimens were also tested by Western blot (Novapath HIV-1 immunoblot; Bio-Rad, Hercules, CA). Specimens positive on EIA and Western blot were considered HIV seropositive. HIV-1 subtyping was performed as previously described.[26,27] EIA-negative HEPS sera were tested for HIV-1 specific IgG and IgA by Western blot. The IgG assay was performed according to the manufacturer specifications (Cambridge-Biotech, Worcester, MA). For HIV-1-specific IgA, the Cambridge Western blot assay was modified as described[29–31] and used with weakly reactive and strongly reactive HIV-positive controls supplied by the manufacturer, a strongly reactive Thai serum sample, and a pool of HIV-1-positive sera. Plasma *gag* RNA was tested with a nucleic acid sequence-based amplification (NASBA) HIV-1 RNA QL kit (Organon Teknika, Durham, NC)[32] and the Roche Amplicor Monitor kit, version 1.5 (Roche Diagnostic Systems, Branchburg, NJ) (sensitivities: 100 and 400 copies/ml plasma, respectively). Detection of plasma protease, integrase, and gp41 RNAs was performed according to a previously described method, which can detect 10 copies of HIV-1 per milliliter of plasma.[33] HIV DNA was detected in PBMCs with a previously described nested *gag* PCR[32] (sensitivity, 2–4 copies per $1.5 \times 10^5$ PBMCs). Syphilis testing was performed with a MACRO-VUE RPR card test (Becton Dickinson Microbiology Systems, Cockeysville, MD) and the Fujirebio TPHA test (Fujirebio, Tokyo, Japan). HSV-2 testing was performed by immunoblotting for antibodies to gG1 and gG2 as described.[34]

### *Immunophenotyping, PBMC isolation, and generation of B lymphoblastoid cell lines*

$CD4^+$ and $CD8^+$ T cell number and percent and natural killer (NK) cell percent were obtained by flow cytometry of whole

blood, using dual-color Simultest monoclonal antibody reagents and flow cytometry instruments according to the manufacturer instructions (Becton Dickinson Immunocytometry Systems, San Jose, CA) and published guidelines.[35] PBMCs isolated from cell preparation tubes (CPTs; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) were cryopreserved and shipped from Thailand to Atlanta, where Epstein–Barr virus (EBV) transformation to generate autologous B lymphoblastoid cell lines (BLCLs) was performed as previously described.[28]

### *HLA typing*

Samples from HIV-negative and HEPS women were HLA typed in Bangkok, using unfrozen peripheral blood lymphocytes, and were analyzed within 24 hr of collection, with a standard two-stage HLA-A and -B microlymphocytotoxicity test as previously described.[36] The presence of the specific HLA-A11 serotype was confirmed at the molecular level with a British Society for Histocompatibility and Immunogenetics (BSHI) HLA class I sequence-specific oligonucleotide probe (SSOP) kit, which identifies but does not subtype all allelic variants of HLA-A*11 (1101-6) as previously described.[19] HIV-positive women and some of the HEPS and control women were additionally typed molecularly for HLA-A*11 status, using primers based on sequences published in the 12th International Histocompatibility Workshop, HLA class I SSP ARMS-PCR Manual or by sequencing as described in Refs. 28 and 37. As HLA sequencing was not performed to confirm possible homozygosity or to resolve blank alleles, HLA-A locus homozygosity was not assessed.

### *Chemokine and chemokine receptor genotyping and function*

CCR5 genotyping was performed according to a modification of the method described by Liu *et al.*[6] and Samson *et al.*[7] Sequencing was performed by standard dideoxy termination techniques, using DNA amplification products as templates. Plasmids containing the CCR5 genes of the FSWs were transfected into QT6 targets and fusion with HIV-1 envelope was tested with HIV-1 subtype B and E envelope glycoproteins as previously described.[38] CCR5 promoter genotyping at positions 59353, 59355, 59402, and 59653 and the CCR2 64V-I substitution were detected according to published methods.[39,40] CCR3 genotyping was performed by restriction fragment length polymorphism, using the restriction endonuclease *Dde*I and gel

TABLE 1. CHARACTERISTICS OF SUBJECTS[a]

| | *HEPS FSWs (n = 14)* | *HIV-infected FSWs (n = 9)* | *Low-risk, HIV-negative (n = 9)* | p *Value*[b] |
|---|---|---|---|---|
| Age, years: mean (range) | 30.4 (21–48) | 28.0 (21–41) | 38.3 (29–54) | 0.4 |
| Ever married | 2 (14.3) | 3 (33.3) | 9 (100) | 0.3 |
| Born in Chiang Rai Province | 14 (100) | 9 (100) | NA | — |
| Thai ethnicity | 14 (100) | 9 (100) | 9 (100) | — |
| Age (years) at first sex work, mean (range) | 17.8 (15–24) | 18.9 (14–25) | — | 0.5 |
| Duration of sex work (years): median (range) | 10 (3–25) | 6 (2–15) | — | 0.2 |
| Brothel-based sex work | 14 (100) | 5 (55.6) | — | 0.01 |
| No. sex partners last day worked at enrollment, mean (range) | 2.3 (1–6) | 3.2 (1–5) | — | 0.2 |
| Amount (Thai baht[c]) client paid for sex, last day worked at enrollment, mean (range) | 305 (75–750) | 624 (90–1500) | — | 0.1 |
| Used condoms <50% of time, last 3 months, at enrollment | 2 (14.3) | 3 (33.3) | — | 0.3 |
| History of injecting drug use | 0 | 0 | — | — |
| History of blood transfusion | 2 (14.3) | 0 | — | 0.5 |
| History of tattoo | 1 (7.1) | 0 | — | 1.0 |
| Oral contraceptive pill use | 6 (42.9) | 5 (55.6) | — | 0.7 |
| DMPA use | 2 (14.3) | 1 (11.1) | — | 1.0 |
| HSV-2 seropositive | 14 (100) | 9 (100) | — | — |
| TPHA seropositive | 14 (100) | 4 (44.4) | — | 0.004 |
| Gonorrhea during cohort study | 4 (28.6) | 5 (55.6) | — | 0.4 |
| Chlamydia during cohort study | 4 (28.6) | 6 (66.7) | — | 0.1 |

*Abbreviations:* HEPS, HIV exposed but persistently seronegative; FSW, female sex worker; DMPA, depot medroxyprogesterone acetate; TPHA, *Treponema pallidum* hemagglutination assay; NA, not available.

[a]Number (percent) of subjects except where noted. —, Information not obtained or not applicable. Data as of April 1996 except where noted. Chi-square and Student *t* tests were used for statistical comparisons.

[b]Comparing HEPS FSWs with HIV-infected FSWs.

[c]U.S. $1 = 25 Thai baht (in 1996).

electrophoresis, and CCR3 sequencing was performed as described above, using amplimers and primers based on published sequences.[41,42] The 3′ G-to-A transition at position 801 in the 3′ untranslated region (3′ UTR) of the stromal-derived factor (SDF-1) gene was determined according to the method described by Winkler *et al.*[43] The RANTES-28G polymorphism in the RANTES promoter was determined as described by Liu *et al.*[44]

### *Generation of HIV-specific cytotoxic T lymphocytes and CTL assays*

Cryopreserved PBMCs were stimulated *in vitro*, using a previously described[11,28] HLA-matched peptide stimulation method. The subtype E/A peptides used were cytotoxic T lymphocyte (CTL) epitopes in previous studies of HIV-1 subtype E/A-infected women from the Chiang Rai FSW cohort and were based on the sequences of 93TH253 for Gag, Pol, and Nef and CM243 for Env as previously described.[28] For HLA-A11-positive subjects, the following CTL epitopes were used: six published CTL epitopes, conserved between subtypes E/A and B or immunoreactive subtype E/A variants of a previously known subtype B epitope[28]; six novel HLA-A11 CTL epitopes identified in HIV-infected women from the Chiang Rai FSW cohort[28,45]; and three potential HLA-A11 CTL epitopes that had weak CTL recognition (<10% lysis) in the previously studied HIV-infected subjects.[28] These 15 peptides were as follows: Gag, 82–91 (IATLWCVHQR); Pol, 248–257 (GIPHPAGLKK), 272–281 (SVPLDESFRK), 313–321 (AIFQSSMTK), 495–505 (QIYQEPFKNLK), 571–579 (FVNTPPLVK), and 894–903 (AVFIHNFKRK); Env, 6–15 (TQMNWPNLWK), 36–46 (VTVYYGVPVWR), 109–117 (ISLWDQSLK), 309–318 (ITVGPGQVFY), 340–348 (RVLKQVTEK), and 762–770 (SLCLFSYHR); and Nef, 73–82 (QVPLRPMTYK) and 83–92 (GAFKLSFFLK). For HLA-A11-negative persons, the following peptides, shown to be CTL epitopes in other HIV-infected Chiang Rai FSWs,[28] were used: HLA-A33: Pol 313–321 (AIFQSSMTK); HLA-A24: Gag 28–36 (KYKMKHLVW) and Env 586–593 (RYLKDQKLL); HLA-A2: Gag 77–85 (SLYNTIATL), Pol 334–342 (VIYQYMDDL) and 464–472 (ILRIPVHGV), Env 191–200 (YRLINCNTSV), 747–755 (RLVSGFLAL), 813–822 (SLLNATAIAV), and 828–836 (KVIEVAQGA), and Nef 136–145 (PLCFGWCFKL) and 180–189 (VLIWKFDSAL). Peptide expansions were performed as previously described,[28] except that cultures were initiated for three subjects at a time, with cryopreserved PBMCs from each HEPS subject cultured concurrently, where possible, with cryopreserved PBMCs from HLA-A locus-matched HIV-positive and HIV-negative controls, all having similar starting numbers of viable PBMCs per vial (known from EBV-B cell generation step). Thus, the cultures were matched for some factors that could influence the rate of expansion of T cells and could be timed to have CTL assays done after the same number of days in culture. After the first CTL assay, some cultures were stimulated again, using irradiated (10,000 rads) autologous BLCLs pulsed with appropriate peptides (10–50 $\mu$M, final concentration) and irradiated (3000 rads) allogeneic normal PBMCs as previously described.[11] CTL assays were performed after 12–23 days, using a standard 5-hr $^{51}$Cr release assay and autologous BLCL targets pulsed (or non-pulsed) with individual peptides or occasionally with peptide pools at a final concentration of 10 $\mu$M as previously described.[28] Spontaneous release was usually less than 20%. HIV-specific lysis was calculated by subtracting the percent lysis ob-

Table 2. HIV-Related Laboratory Studies[a]

| | *HEPS FSWs* | *HIV-infected FSWs* | *Low-risk, HIV negative* |
|---|---|---|---|
| Positive HIV-1 EIA | 0/14 | 9/9 | 0/9 |
| Mean no. (range) of EIA tests/person | 20 (4–30) | 1 (1) | 1 (1) |
| Subtype E peptide EIA positive | ND | 9/9 | ND |
| Total Ig HIV-1 WB positive | 0/9 | 9/9 | ND |
| IgG HIV-1 WB positive | 0/12 | ND | ND |
| IgA HIV-1 WB positive | 0/12 | ND | ND |
| Plasma *gag* RNA, qualitative test positive | 0/9 | ND | ND |
| Plasma *gag* RNA, quantitative test positive | 0/9 | 9/9 | 0/9 |
| Plasma gp41 PCR positive | 0/12 | ND | ND |
| Plasma protease PCR positive | 0/12 | ND | ND |
| Plasma integrase PCR positive | 0/12 | ND | ND |
| PBMC *gag* DNA positive | 0/7 | ND | ND |
| $CD4^+$ cells/$\mu$l, mean (range) | 1154 (670–2800) | 346 (23–576) | 724 (490–889) |
| $CD4^+$ cell percent, mean (range) | 43.1 (27–53) | 18.1 (3–36) | 38.7 (35–46) |
| $CD8^+$ cells/$\mu$l, mean (range) | 641 (260–1260) | 913 (410–1724) | 473 (309–664) |
| $CD8^+$ cell percent, mean (range) | 23.7 (15–39) | 52.2 (41–66) | 25.7 (14–32) |
| NK cell percent, mean (range) | 14.2 (7–25) | 12.6 (6–18) | 16.2 (12–27) |

*Abbreviations:* EIA, Enzyme immunoassay; FSW, female sex worker; WB, Western blot; PCR, polymerase chain reaction; NK, natural killer; ND, not determined.

[a]Immunophenotyping data for HEPS and HIV-infected FSWs from 1996 (or earlier) specimens. Low-risk HIV-negative data from 1998.

tained from non-peptide-pulsed targets from the percent lysis of peptide-pulsed targets. A positive CTL response was defined as HIV-specific lysis ≥10% observed at ≥1 effector-to-target (*E*:*T*) ratio. If the lysis was observed only at the lowest *E*:*T* ratio, it was considered a negative response.

### *HIV antigen and mitogen-induced proliferation and chemokine production*

Cryopreserved cells were tested for HIV antigen- and mitogen-induced proliferation essentially as described previously.[29] For gp120 proteins, 5 μg/ml each of LAV (subtype B) and Chiang Mai (subtype E) gp120s (both from Protein Sciences, Meriden, CT) and 10% interleukin 2 (IL-2; ABI, Columbia, MD) was used. For Gag, a 1:1 mixture of p24 (IIIB) and 5 μg/ml of a p24 peptide (PVHAGPIAPG, 5 μg/ml; Alpha Diagnostic International, San Antonio, TX) and 10% IL-2 were used. This p24 peptide was chosen because it has been demonstrated previously to be immunoreactive in HIV-1-infected persons.[46] Two of the six women in each group lacked sufficient cell numbers, so their PBMCs were combined for antigen exposure. Supernatants were collected on day 6 and stored at −70°C for analysis of chemokine production.

### *Cytokine and chemokine assays*

Frozen supernatants from the initial pooled peptide-expanded CTL cultures, from some of the restimulated CTL cultures from three test groups (harvested on days 3, 7, 10, and 14 after initial peptide stimulation), and from the proliferation cultures were tested by enzyme-linked immunosorbent assay (ELISA), using commercial kits: macrophage inflammatory protein 1α (MIP-1α), MIP-1β, tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), soluble TNF receptor II (TNF-RII), and RANTES (R&D Systems [Minneapolis, MN] or Biosource International [Camarillo, CA]). Supernatants from CTL cultures were also tested for soluble HIV-suppressive activity as described.[47]

### *Statistical analyses*

For analysis of discrete variables, $\chi^2$ tests were performed unless a cell number was <5, in which case a two-tailed *p* value was obtained by the Fisher exact test. The Student *t* test was used for comparison of means and the Wilcoxon rank sum test was used for comparison of medians, using SAS version 6.12 (SAS, Cary, NC) and EpiInfo version 6.04 (CDC, Atlanta, GA).

## RESULTS

### *Clinical and demographic characteristics of study population*

The demographic and HIV exposure factors of the 14 HEPS women and the 9 HIV-infected FSWs who were available for HLA typing are described in Table 1. Because of limited sample availability in these groups, not all women were tested in every genetic and immunologic study. All HEPS and HIV-positive women were ethnically Thai and were natives of Chiang Rai Province. The two groups were also similar in age and had started sex work at similar ages. By definition, all HEPS women worked in brothels, while only five of the nine HIV-infected FSWs were brothel workers; the other four HIV-infected women had worked in other establishments where the cost of sexual services was higher. Both groups reported having at least

Table 3. Frequencies of HLA and Chemokine Receptor or Chemokine Genes[a]

| *Genotype* | *HEPS FSWs* | *HIV-infected FSWs* | *Low-risk, HIV negative* |
|---|---|---|---|
| HLA-A11 | 12/14 (86) | 4/9 (44) | 6/9 (67) |
| HLA-A3 supertype | 13/14 (93) | ND | 8/9 (89) |
| CCR5 Δ32 | 0 | 0 | 0 |
| CCR5 59402 promotor genotype | | | |
| GG | 4/7 (57) | 2/7 (29) | 3/9 (33) |
| GA | 3/7 (43) | 5/7 (71) | 4/9 (44) |
| AA | 0/7 (0) | 1/7 (14) | 2/9 (22) |
| CCR2 190 genotype | | | |
| AA | 1/11 (9) | 0/12 (0) | 0/9 (0) |
| AG | 3/11 (27) | 3/12 (25) | 2/9 (22) |
| GG | 7/11 (64) | 10/12 (83) | 7/9 (78) |
| SDF-1 3′ UTR 801 genotype | | | |
| AA | 1/12 (8) | 0/9 (0) | 0/7 (0) |
| AT | 6/12 (50) | 3/9 (33) | 2/7 (29) |
| TT | 5/12 (42) | 6/9 (67) | 5/7 (71) |
| RANTES-28G genotype | | | |
| CC | 7/7 (100) | 8/10 (80) | 7/9 (78) |
| CG | 0/7 (0) | 2/10 (20) | 1/9 (11) |
| GG | 0/7 (0) | 0/10 (0) | 1/9 (11) |

[a]Numbers in parentheses represent percentages. HLA-A frequencies are reported as phenotypes, as not all were sequenced. CCR5 promotor numbering and notation are based on GenBank accession number U95626, except for 59402, for which the genotype notation is in reverse.

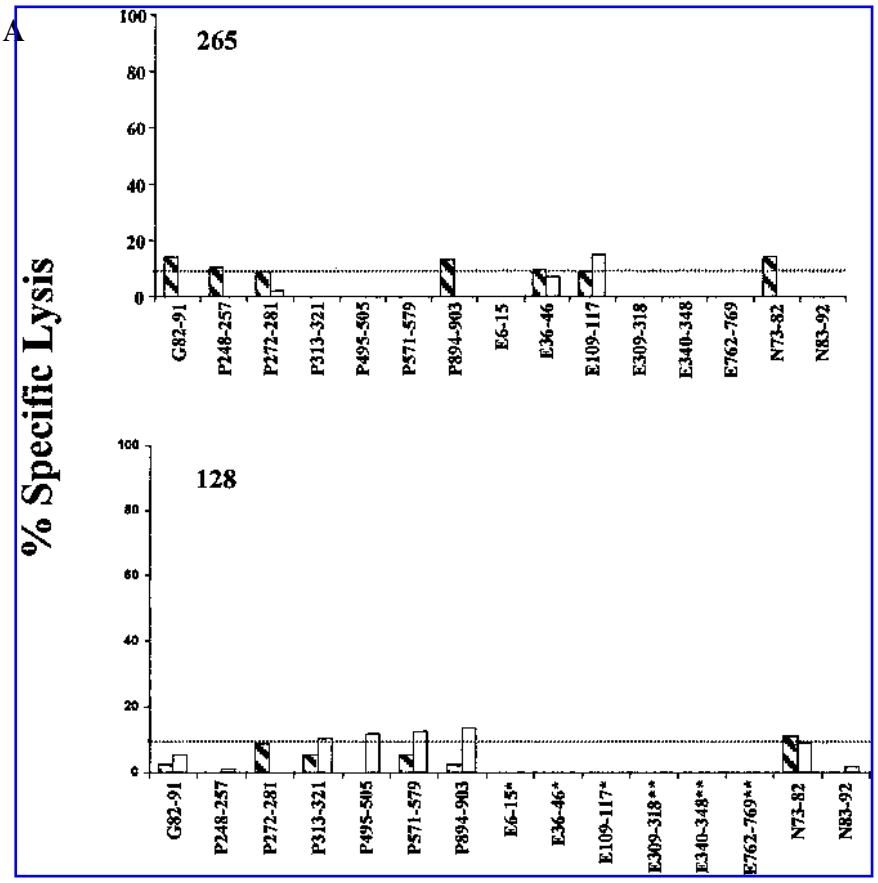


**FIG. 1.** HIV-specific CTL response in HEPS and HIV-positive individuals. PBMC cultures from subjects with indicated ID numbers were stimulated with peptide pools and tested against BLCL targets pulsed with individual peptides or peptide pools. Effector to target ratios included 90:1 (filled bars), 60:1 (horizontal stripes), 30:1 (hatched bars) and 10:1 (open bars). HEPS HLA types (in **A** and **B**) were: 265, A2/A11; 128, A11/33; 186, A2/11; 256, A11/33. (**A**) CTL data in two HEPS individuals following one *in vitro* stimulation; Env peptide pools *(6–15, 34–44 and 109–117) or **(314–323, 346–356, 761–769) were used where indicated (**B**) CTL data in four HEPS individuals following a second *in vitro* stimulation; Env peptide pools *(6–15 and 36–46) or **(340–348, 762–770) were used where indicated. (**C**) CTL data in four representative HIV positive persons. HLA types were: 176, A11; 184, A11; 125, A2/7; 144, A2. Env peptide pools *(6–15, 36–46 and 109–117) or **(309–318, 340–348, 762–770) were used.

two partners per day. HEPS women had an average of 10 years of sex work, but one women had worked in commercial sex for 25 years. Thus, most subjects had been FSWs entirely during or for some part of the Thai HIV-1 epidemic. Both groups of women reported similar contraceptive histories. All HEPS and HIV-positive FSWs had serological evidence of HSV-2 infection, and all HEPS women, by definition, had serological evidence of prior syphilis. Compared with the HEPS women, the HIV-infected control group had somewhat higher rates of gonorrhea and chlamydia cervicitis during study follow-up. Compared with these two groups, the nine low-risk HIV-negative women were older and all were married and of Thai ethnicity.

### *HEPS women have multiple negative serologic and molecular HIV tests and normal immunophenotypes*

Analysis of HIV-1-specific antibodies in multiple samples obtained from the HEPS women over at least 3 years revealed no evidence of HIV infection (Table 2). Since studies have suggested the presence of HIV-1-specific IgA antibodies in seronegative partners of HIV-infected persons,[31] we tested for IgG and IgA antibodies. Again, no HIV-1-specific antibodies could be identified in any HEPS subject, whereas all HIV-1-infected women had HIV-1 antigen-specific bands (data not shown). Tests of plasma for HIV-1 RNA in multiple regions including p24, protease, integrase, and *env* and at multiple times were negative, and proviral HIV-1 DNA was absent in PBMCs from seven women, three of whom were studied for HIV-specific T cell responses (see below). Insufficient sample was available to test all HEPS subjects for PBMC proviral DNA. As expected, $CD4^+$ cell counts and percents were lower and $CD8^+$ counts and percents were higher in the HIV-infected control women (Table 2) than in HEPS women. Immunophenotyping data were available for 152 HIV-negative women in the Chiang Rai STD cohort. $CD4^+$ and $CD8^+$ T cell numbers and percents and NK percents of HEPS women were not significantly

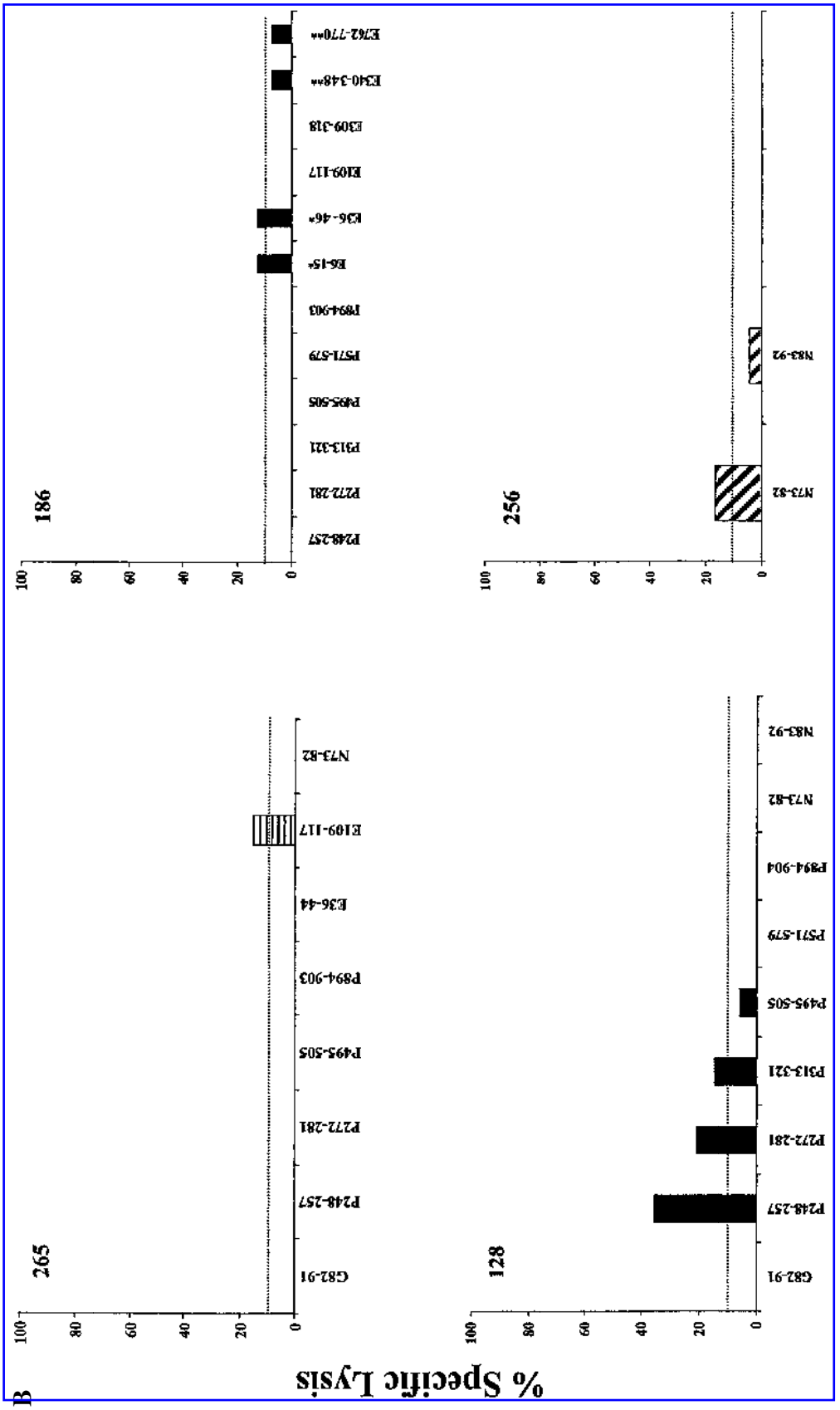


FIG. 1. *Continued.*

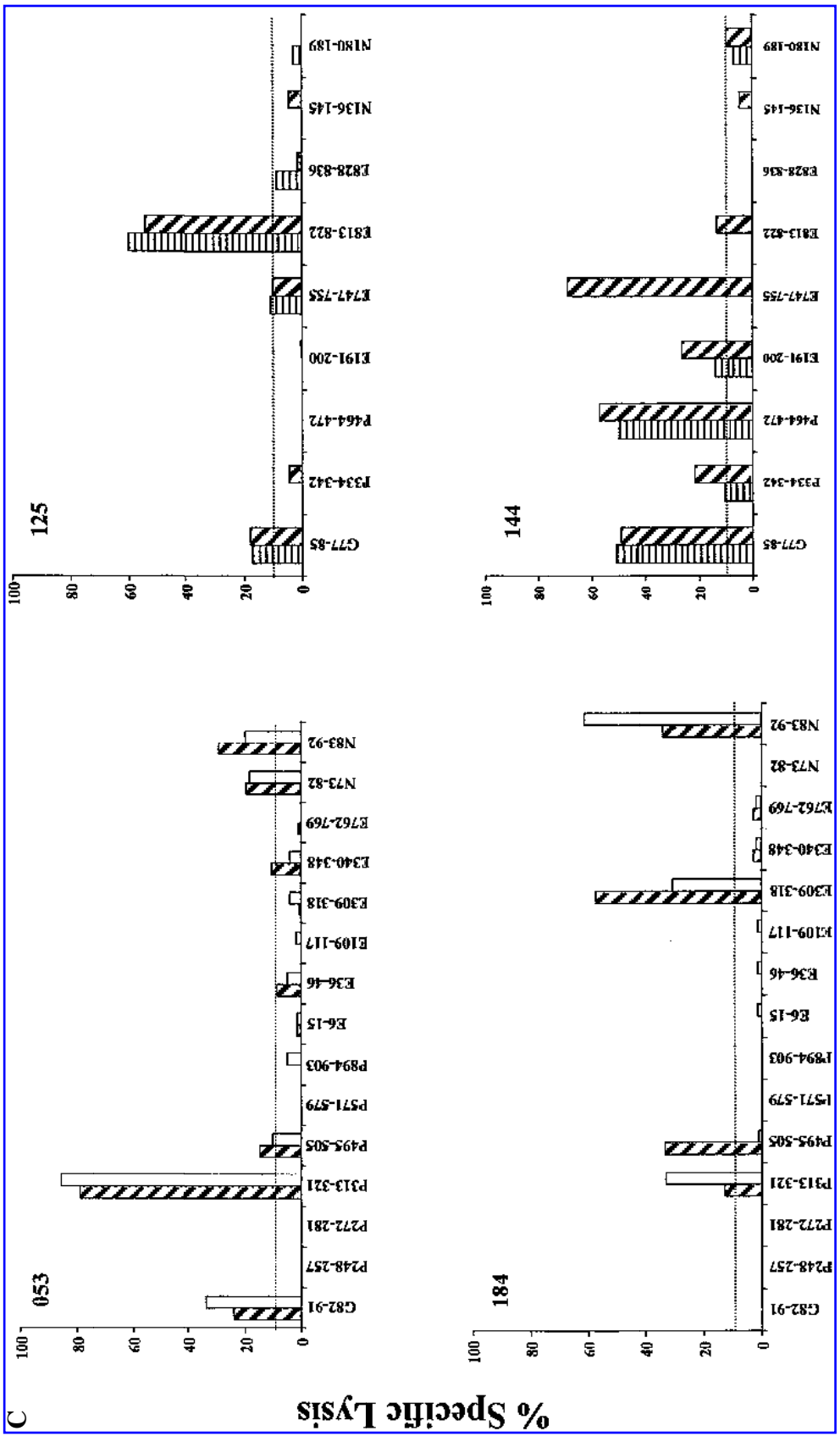


FIG. 1. *Continued.*

different from those of non-HEPS, non-HIV-infected cohort subjects (data not shown).

### *HLA studies*

Serologic class I MHC typing, using reagents appropriate for Thai populations, was performed for all HEPS and HIV-negative control subjects. HIV-positive women were typed by molecular methods. The frequency of the HLA-A11 serotype and the HLA-A*11 genotype was increased in HEPS FSWs (86%; Table 3) compared with the HIV-positive and HIV-negative groups and with published northern Thai healthy population control frequencies (56%, obtained in the 1970s by Greiner *et al.*,[48] that is, before the onset of the Thai HIV epidemic): $\chi^2 = 4.6$, $p = 0.03$. Molecular HLA typing of the HLA-A11-positive subjects in all three groups determined that all were HLA-A*1101 positive except for subject 186, who had the HLA-A*1103 subtype. A high frequency of the HLA-A3 supertype was found in all three groups.

### *Cytotoxic T cell studies*

HIV-specific CTL responses were detected in cultures from four of seven (57%) HEPS women but in none of the HIV-negative controls ($p = 0.019$; Fig. 1A and B, and Table 4). In both groups HLA-A locus-specific subtype E/A CTL epitopes were used to stimulate the cryopreserved PBMCs after thawing. The CTL responses were generally weak (<15% specific lysis) in most HEPS women, although after a second stimulation with the same pool of peptides, lysis levels then increased up to almost 40% in some women (e.g., subject 128; Fig. 1A and B) but not in all. Two HLA-A11-positive HEPS women (subjects 128 and 265) had CTL responses to one or more peptides after one *in vitro* stimulation (Fig. 1A), while two additional HLA-A11-positive HEPS women (subjects 186 and 256) had CTL responses only after a second stimulation. In the four HLA-A11-positive HEPS women (subjects 128, 256, 265, and 186; Fig. 1A and B) positive CTL responses were seen to Nef, Pol, Env, and Gag, with Nef responses dominating (three of four, 75%) over both Env (two of four, 50%) and Pol (two of four, 50%) responses, Gag responses were observed only in one HEPS woman (subject 265, to Gag 82–91, IATLWCVHQR). However, only one Gag peptide was included in the panel of HLA-A11 epitopes used. In Nef, all three responses (subjects 128, 265, and 256) were to the same Nef peptide, 73–82 (QVPLRPMTYK). In Pol, peptide 248–257 (GIPHPAGLKK) was recognized by two women (subjects 265 and 128, after restimulation) while Pol 272–281 (SVPLDESFRK), 313–321 (AIFQSSMTK), and 495–505 (QIYQEPFKNLK) were exclusively recognized by HEPS woman 128, two of these after a second *in vitro* stimulation. Donor 265 recognized Pol 894–903 (AVFIHNFKRK). Env responses were to Env 109–117 (donor 265 after two restimulations) and to a pool containing Env 6–15 (TQMNWPNLWK) and Env 34–44 (VTVYYGVPVWR) in donor 186 after restimulation.

All HIV-positive FSWs tested had CTL responses to one or more peptides (Table 4). Some donors recognized many peptides, while others had responses restricted to one or a few peptides from one HIV protein region.[28] Data from four representative subjects are shown (Fig. 1C). Most noticeable is the higher level of lysis observed in these HIV-positive women, compared with the HEPS individuals. No CTL activity was detected in any of the 9 unexposed HIV-negative controls after one *in vitro* expansion of PBMCs with HLA-A locus-matched subtype E/A CTL epitopes. Because PBMCs were restimulated *in vitro* in seven of the HEPS women, we also restimulated PBMCs in two HLA-A11-positive, HIV-negative women with all HLA-A11 HIV CTL epitopes. No CTL activity was detected in these restimulated cultures.

Supernatants from the peptide-stimulated CTL cultures were tested for production of IFN-$\gamma$, TNF-$\alpha$, and soluble TNF-RII and for their ability to suppress HIV, using an *in vitro* HIV replication assay.[47] No differences between the HEPS women and the HIV-positive or -negative controls were seen at any time point, and cultures that were positive in CTL assays did not have different levels of these cytokines or suppressive activity compared with those of cultures that were negative (data not shown).

TABLE 4. HIV-SPECIFIC CTLs IN HEPS AND HIV-POSITIVE SUBJECTS[a]

| | *HEPS FSWs (n = 7)* | *HIV-infected FSWs (n = 8)* | *Low risk, HIV negative (n = 9)* |
|---|---|---|---|
| Number CTL positive | 4/7 (57) | 8/8 (100) | 0 |
| CTL lysis >30% | 1/4 (25) | 6/8 (75) | |
| CTL lysis 10–30% | 3/4 (75) | 2/8 (25) | |
| Mean number (range) of peptides recognized | 2 (1–5) | 3.75 (1–9) | |
| Response to one peptide | 1/4 (25) | 1/8 (12) | |
| Response to two or more peptides | 3/4 (75) | 7/8 (88) | |
| Env-reactive CTLs | 2/4 (50) | 6/8 (75) | |
| Gag-reactive CTLs | 1/4 (25) | 5/8 (63) | |
| Pol-reactive CTLs | 2/4 (50) | 6/8 (75) | |
| Nef-reactive CTLs | 3/4 (75) | 4/8 (50) | |

[a]Numbers in parentheses represent percentages unless otherwise indicated.

*Proliferative and chemokine responses to mitogens and HIV proteins*

Analysis of HIV-specific and mitogen-induced proliferative responses in PBMCs from a subset of patients (from a different time point than that of the CTL studies) revealed that all groups had similar responses to phytohemagglutinin (PHA) while the HIV-negative group had a better proliferative response to pokeweed mitogen (PWM) than did either the HIV-positive group ($p = 0.04$) or the HEPS group ($p = 0.06$) (Fig. 2A). No HIV-1-specific gp120 or $p^{24}$ *gag*-specific proliferative responses were observed in the HEPS women, or the HIV-negative or -positive women. The lack of proliferation in the HIV-positive women is not unexpected, as proliferative helper T cell (Th) responses in HIV-infected persons are typically observed only in some HIV-positive subjects, using this assay in our lab-

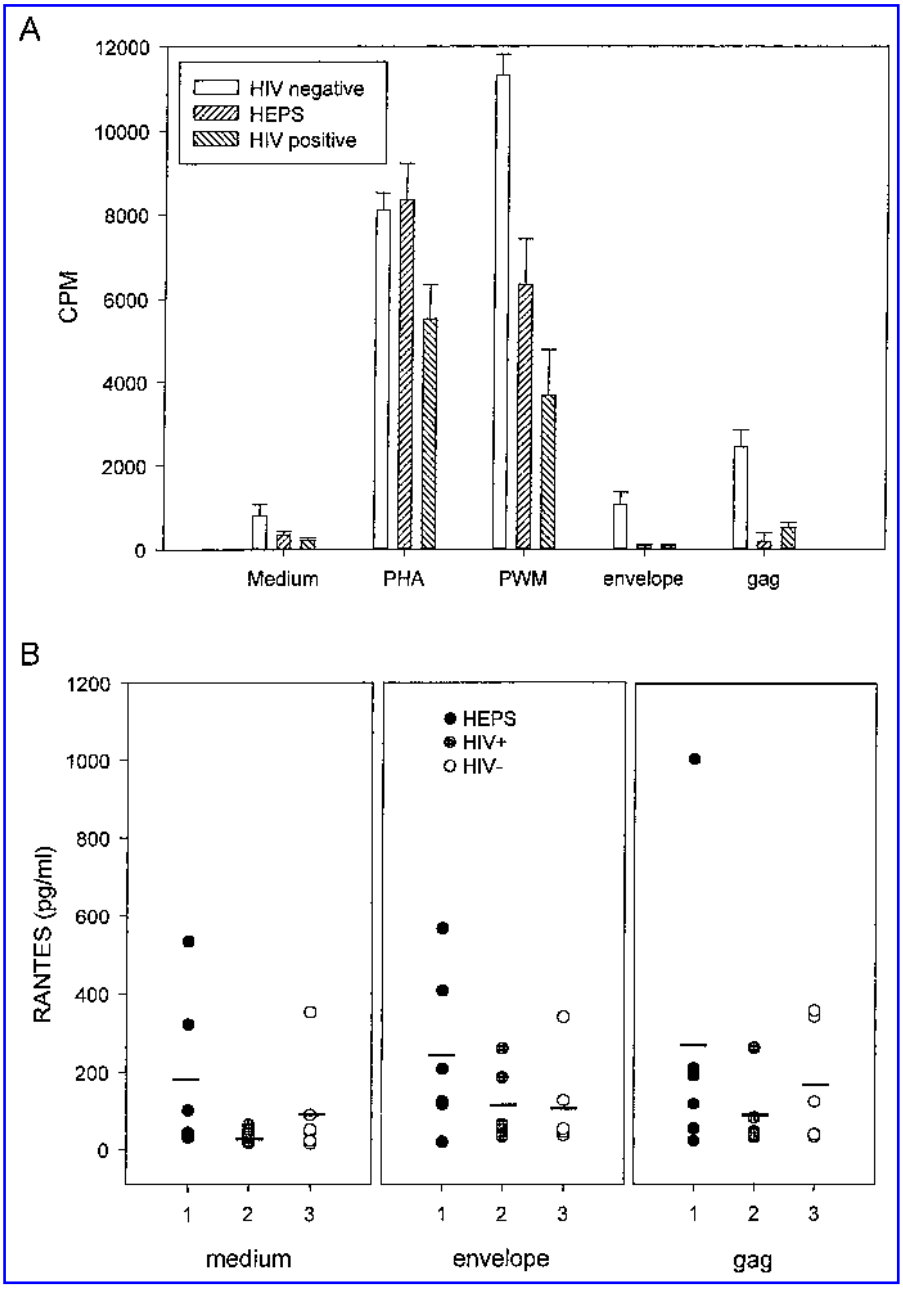


**FIG. 2.** Proliferative responses and RANTES production. PBMCs from indicated groups were cultured in medium alone or stimulated with mitogens (PHA, PWM) or HIV-1 envelope (subtype B and E) or gag (p24 and p24 peptide) as described in materials and methods. The data from individuals whose PMBCs had combined env/gag stimulation are presented in both the gag and env columns. (**A**) Proliferative responses, measured by incorporation of [$^3$H]TdR on day 7 of culture. (**B**) RANTES levels in supernatants from proliferation cultures, measured on day 6, using ELISA as described. The data are presented as cpm ([$^3$H]TdR) or pg/ml (RANTES) with the mean average ± SEM of six persons in each group.

oratories,[29] and after acute infection or in long-term nonprogressors[46] in other studies.

To determine whether endogenous or antigen-specific production of chemokines could account for HIV resistance among the HEPS women, supernatants from these cultured PBMCs were tested for RANTES production (Fig. 2B). Mean endogenous RANTES levels were somewhat higher in the HEPS women when compared with the HIV-negative controls, and although the differences in the medians of the groups were not significant, two HEPS women had RANTES levels >2-fold over the median observed (100 pg/ml) in HIV-negative controls and some of the highest levels of RANTES were observed in several HEPS women. No differences in the level of MIP-1$\alpha$ or -$\beta$ were observed (data not shown). Levels of these chemokines in response to gp120 or p24 stimulation were also comparable in all three groups, although a trend for higher levels of RANTES in the HEPS women compared with other groups was observed.

### *Chemokine receptor and chemokine gene-related studies*

Analysis of the CCR5 genes of the HEPS FSWs indicates absence of the 32-bp deletion in all 14 women (Table 3) and an absence of missense, nonsense, or any other polymorphisms in full-length CCR5 sequences in 7 FSWs. No significant differences in the distribution of the four previously described CCR5 promoter polymorphisms[40] between the three groups was observed; however, all HEPS women had one or more copies of the 59402 allele, associated with reduced perinatal HIV transmission,[49] and 57% were homozygous for this allele compared with 29% of the HIV-positive controls ($p = 0.6$) or 33% of the HIV-negative controls ($p = 0.6$). The frequency of the CCR2 position 190 polymorphism, that generates the 64V-I amino acid change associated with delayed HIV disease progression, was similar in the HEPS women compared with the controls. In all three groups, the expected 100% linkage between CCR2 64I and CCR5 promoter position 59653T was observed. Full-length nucleotide sequencing of the CCR3 gene in three HEPS FSWs revealed sequences identical to those reported by Combadiere *et al.*[41] and Daugherty *et al.*,[42] which have a serine at position 276. These sequences differ from that reported by Ponath *et al.*, which has a threonine at this position.[50] HEPS individuals tended to have higher SDF-1 3′ UTR 801A allele frequencies (50%) than did the HIV-positive (33%, $p = 0.4$) or HIV-negative (29%, $p = 0.3$) controls. Homozygosity for this allele was observed in one HEPS woman and in none of the controls. Because of the high endogenous RANTES levels in some HEPS women, we examined the prevalence of the RANTES-28G polymorphism that is associated with increased RANTES transcription.[44] The frequency of this allele was similar in all three groups.

The function of the CCR5 gene in five HEPS individuals was examined in a fusion assay, in which HeLa cells were infected with recombinant vaccinia viruses expressing envelope glycoproteins from an envelope subtype E virus, CM243, or from two subtype B viruses, JR-FL and 89.6 (Fig. 3). CM243 was isolated in northern Thailand[51]; its envelope requires CCR5 for fusion and no other HIV-entry cofactors (R.W. Doms, unpublished data). JR-FL is a macrophage-tropic virus, and 89.6 has dual macrophage and T cell tropism.[38] The fusion levels observed for the cloned HEPS CCR5 genes with all three envelopes were equivalent to those of the control gene, suggesting that HEPS CCR5 receptors can promote HIV entry of divergent isolates, including strains from subtypes B and E. This was confirmed in an acute infection assay with subtype B and E viruses.[47]

## DISCUSSION

We describe 14 HEPS women who remain persistently HIV negative by PCR and serologic assays despite at least 3 years

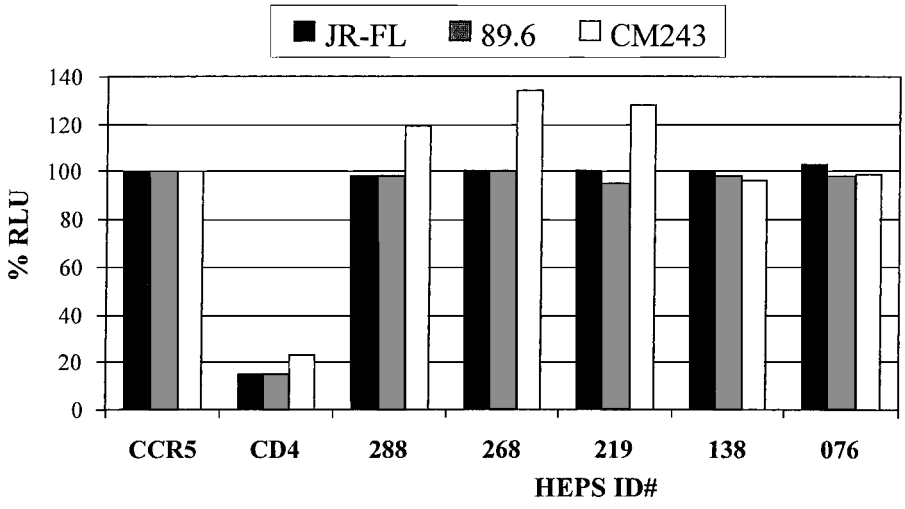


**FIG. 3.** HIV env-fusion studies of CCR genes in 5 HEPS FSWs. Quail QT6 cells expressing luciferase under control of the T7 promoter and CD4, or CD4 in combination with wild type CCR-5 or CCR-5 clones obtained from 5 HEPS individuals were mixed with HeLa cells expressing the indicated HIV-1 env proteins and T7 polymerase. After 8 h, the cells were lysed and luciferase activity measured in relative light units (RLU).

TABLE 5. MULTIPLE FACTORS POSSIBLY ASSOCIATED WITH THE HEPS PHENOTYPE[a]

| | | *Genetic factors (no. of copies of allele)*[b] | | | *Immunologic factors* | |
|---|---|---|---|---|---|---|
| *HEPS ID no.* | *Years of FSW*[c] | *HLA-A11 positive (HLA-A type)* | *CCR5P 402G* | *SDF-1 3′UTR 801A* | *CTLs* | *Elevated basal RANTES*[d] |
| 128 | 11.0 | + (11/33) | 2 | 1 | + | − |
| 186 | 25.1 | + (2/11) | 1 | 1 | + | + |
| 212 | 3.5 | + (2/11) | 2 | 0 | − | + |
| 256 | 8.6 | + (11/33) | 2 | 0 | + | − |
| 265 | 9.6 | + (2/11) | 2 | 1 | + | |
| 317 | 12.8 | + (11/−) | 1 | 1 | − | |
| 340 | 9.1 | − (24/33) | 1 | 0 | − | − |
| 076 | 14.4 | + (2/11) | | 2 | | |
| 219 | 10.3 | + (11/−) | | 1 | | |
| 268 | 9.7 | − (2/−) | | 0 | | |
| 288 | 4.1 | + (11/29) | | | | |
| 048 | 5.9 | + (2/11) | | 1 | | + |
| 138 | 15.4 | + (11/33) | | 0 | | |
| 322 | 4.6 | + (11/33) | | | | |

[a]Not all women were tested for these factors, as explained in Materials and Methods. Blank entries indicate assay not done.
[b]CCR5 promotor (CCR5P) and SDF-1 3′ UTR DNA base numbers; subject 186 typed as HLA-A*1103 by sequencing.
[c]No correlation between the duration of sex work and presence or absence of CTLs or the number of peptides recognized in CTL assays was noted (data not shown).
[d]RANTES level >100 pg/ml, the average level observed in HIV-negative controls.

of high HIV exposure. Although samples from all these women could not be tested for all potentially HIV-protective factors, several T cell-related and chemokine-related factors that could contribute to HIV resistance were identified (Table 5). The HIV-specific cytotoxic T cell activity in frozen PBMC specimens from HEPS women, in the absence of any similar activity in samples from control HIV-negative women, confirms that the HEPS women have been HIV exposed. Moreover, these data, in the absence of detectable HIV, suggest that CTLs may have contributed to protection by clearing HIV-infected cells. The high frequency of HLA-A11 in the HEPS women suggests it could play a direct role in T cell-mediated protection or, alternatively, could reflect other genetic or linked protective mechanisms. Several HEPS women also had high endogenous production of RANTES and a higher prevalence of some chemokines receptor genotypes previously known to reduce HIV infection. Thus T cell-related and chemokine-related host factors may have synergized to prevent HIV infection or spread.

HIV-specific CTLs have been found in multiple previous studies of HEPS individuals from the commercial sex trade or other high-exposure settings.[11,13–15,17,18,20,22,23] The combined data from these studies suggest that the ability of CTLs to kill HIV-infected cells is responsible for a component of the HEPS phenotype and therefore may be contributing to HIV resistance in our Thai HEPS subjects. Findings of CTLs in cervical vaginal lavage (CVL) specimens of HEPS FSWs from Nairobi[52] and in uninfected women in HIV-discordant couples[53] suggest that in HEPS women, their presence at the site of HIV exposure may be particularly important in preventing infection. Alternatively, the CTLs observed in our and other HEPS individuals could reflect a compartmentalized infection, as observed in some HEPS individuals,[54] in which their role may be to contain a localized HIV infection.

It is not known what level of HIV-specific CTLs in the blood or in a mucosal site is HIV protective. In this study, the observed CTL lysis levels were low, although in some cases CTL activity was detected at effector-to-targetratios of ≤30:1. Although quantitative techniques were not used, the low CTL lysis levels suggest that the number of circulating CTLs in these women was low. Alternatively, while other studies have noted higher CTL lysis levels in HEPS individuals, these studies were performed with fresh PBMCs while we were limited to working with cryopreserved PBMC. Thus, the lytic levels observed in this study may underestimate the *in vivo* CTL frequency in the Chiang Rai HEPS FSWs. It is also possible that higher signals could have been obtained in our study by using an alternative assay such as a peptide ELISpot assay based on cytokine production, or by using MHC tetramers loaded with a frequently recognized peptide such as Nef 73–82. Moreover, CVL samples, if available, might have had higher CTL frequencies. In other studies using fresh samples and sensitive assays such as a cytokine ELISpot, the estimated frequency of HIV-specific antigen-specific T cells in HEPS PBMCs or CVL is typically less than 1/3000 PBMCs.[15,52,55,56] This is lower than that typically observed in many HIV-infected persons and near the detection limits of some CTL assays.[55,57,58] An increase in CTLs with increasing duration of exposure has been observed in one HEPS study,[55] while loss of CTL responses has been noted in HEPS FSWs who have discontinued sex work (and who may subsequently become HIV infected[59]). We observed no association between the presence of CTLs and duration of sex work (Table 5), although this interpretation may be limited by the small sample size.

Characterization of the differences between CTLs in HEPS individuals and HIV-infected individuals may provide clues as to the nature and specificity of protective responses and may be useful for vaccine design. Compared with HIV-infected FSWs, CTL responses to Nef dominated in HEPS FSWs and were to a more restricted range of peptides. Two peptides, Pol 894–903 and Env 109–117, were uniquely recognized by HEPS individuals (Table 4). These data are consistent with findings from some previous studies identifying frequent Nef-directed, as well as Pol-, Gag-, and Env-directed, CTLs in HEPS individuals.[11,15,16,56] They are consistent with some evidence that the repertoire of peptides recognized by HEPS individuals and by HIV vaccine recipients may be different from those recognized by HIV-infected persons.[49,55] The finding of a more restricted repertoire of peptides in HEPS individuals, as found in this study, could indicate a response focused on conserved regions that can repetitively clear HIVs from different infecting partners during multiple exposures. It could also be interpreted to support the finding that these women are not HIV infected, as diversity of the CTL response in HIV-infected persons can reflect exposure to evolving infecting isolates.

The elevated frequency of HLA-A11 in these HEPS women compared with frequencies obtained from northern Thai control subjects before the HIV epidemic suggests that this allele or linked genes may play a role in HIV protection. In limited analyses we found no evidence to suggest that other HLA class I alleles or other linked genes on chromosome 6 (TNF, HLA-DRB, HLA-DQB, or TAP) were HEPS associated (data not shown). It is interesting to note that a high frequency of HLA-A11 was also noted in the HEPS FSWs from the nearby city of Chiang Mai.[19] If these two HEPS FSW groups are combined, the HLA-A11 frequency compared with northern Thai controls (56%) is significantly elevated ($p = 0.01$). A plausible explanation may be that the unique propensity of this allele to select peptides with positively charged C-terminal anchor residues[60] influences the epitope specificity of CTL response to HIV that is generated. Indeed, similar structural mechanisms have been invoked for the observed protective associations of HLA-A2-related alleles with HIV transmission in another HEPS cohort.[21] Alternatively, other antigen-specific or non-antigen-specific immune or genetic factors may interact with HLA-A11 to influence qualitative or quantitative aspects of the CTL response such as the efficiency of T cell recruitment, kinetics, or capacity to generate a memory response.

One link between antigen- and non-antigen-specific antiviral responses may relate to the finding in some HEPS women of high basal RANTES levels in circulating PBMCs and the additional observation that CD4-monocyte cocultures of some HEPS women produce a novel soluble HIV-suppressive factor.[47] $CD8^+$ T cells express receptors for several chemokines, including RANTES, which influence migration of $CD8^+$ T cells. More importantly, by binding to CCR3 and upregulating Fas ligand, RANTES markedly enhances CTL lysis of HIV-specific MHC-restricted $CD8^+$ T cells.[61,62] RANTES and other $\beta$-chemokines also directly inhibit HIV infection of PBMCs in a subtype-independent fashion.[63,64] Of the three chemokines tested in these studies, RANTES had the most potent inhibitory activity[64]; in addition to its protective effect in animal models of HIV, it has been shown to protect against HIV-1 infection in hemophiliac patients.[65–67] The high RANTES production observed in some of the HEPS women in this study could contribute to protection by reducing the ability of CCR5-tropic HIV strains to enter cells.

Genetic HIV resistance in this cohort could not be attributed to the CCR5 $\Delta 32$ deletion, as this was not observed in the Chiang Rai HEPS women. This finding was not unexpected, as the frequency of this polymorphism is low in noncaucasoid and Thai populations[5,68,69] and their PBMCs were readily infectable with subtypes B and E HIV.[47] However, HEPS women had a slightly higher frequency of two other genes associated with reduced HIV transmission. The CCR5 59402GG promoter that was more prevalent in HEPS women is significantly associated, in perinatally HIV-exposed children, with reduced maternal–infant HIV transmission.[39] The SDF-1 3′ A allele, which was more prevalent in the HEPS women, has been reported to be associated with HIV resistance in highly exposed males from the U.S. Multicenter AIDS Cohort Study.[43] While the effects of these polymorphisms on the structure or function of CCR5 or SDF-1 are not yet known, possible mechanisms for these associations could include effects of CCR5 promoter types on transcription or translation of CCR5, while SDF-1 polymorphisms could affect the function of this chemokine in blocking the entry of T-tropic strains through CXCR4. Indeed, it has been shown that binding of SDF to CXCR4 results in rapid downregulation of the receptor and enhanced inhibition of HIV-1.[70,71]

In summary, while all subjects in this study were not tested for all of these potentially protective factors, most HEPS women had one or more of the factors and several women had evidence of an additional novel soluble suppressive factor.[47] These data, taken together with findings from previous HEPS studies, suggest that the mechanisms of HIV resistance may be multiple. In an individual or population they may be independent, additive, or multiplicative. The finding of several different genetic or immunologic factors in our study and in other HEPS groups emphasizes the need for additional studies of similar populations and for enlargement of HEPS groups from genetically similar backgrounds to continue to elucidate the factors that impact HIV transmission, such as vaccines. Our study, for example, could suggest that components of a preventive HIV vaccine should be designed to induce HIV-specific CTLs, should possibly include Nef, and should elicit production of $\beta$-chemokines including RANTES.

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