Research Article

HIV-1-specific cellular immune responses among HIV-1-resistant sex workers

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Summary  The goal of the present study was to determine whether there were HIV-1 specific cellular immune responses among a subgroup of women within a cohort of Nairobi prostitutes (n = 1800) who, despite their intense sexual exposure to HIV-1, are epidemiologically resistant to HIV-1 infection. Of the 80 women defined to be resistant, 24 were recruited for immunological evaluation. The HIV-1-specific T-helper responses were determined by IL-2 production following stimulation with HIV-1 envelope peptides and soluble gp120. Cytotoxic T lymphocyte responses were determined by lysis of autologous EBV-transformed B cell lines infected with control vaccinia virus or recombinant vaccinia viruses containing the HIV-1 structural genes env, gag and pol. Resistant women had significantly increased HIV-1 specific T-helper responses, as determined by in vitro IL-2 production to HIV-1 envelope peptides and soluble glycoprotein 120, compared with low-risk seronegative and HIV-1-infected controls (P < 0.01, Student’s t-test). Seven of the 17 (41%) resistant women showed IL-2 stimulation indices ≥ 2.0. HIV-1-specific CTL responses were detected among 15/22 (68.2%) resistant women compared with 0/12 low-risk controls (Chi-squared test, P < 0.001). In the two resistant individuals tested, the CTL activity was mediated by CD8+ effectors. Many HIV-1-resistant women show evidence of HIV-1-specific T-helper and cytotoxic responses. These data support the suggestion that HIV-1-specific T-cell responses contribute to protection against HIV-1 infection.

Key words: Africa, AIDS, cellular immunity, cytotoxic T lymphocyte, exposed uninfected, HIV, resistance, T helper cell, vaccine.

Introduction

The continued global spread of HIV-1, despite the efforts of public health programs, and the inaccessibility of new treatment options to more than 90% of the world’s infected1 make the development of HIV-1 vaccines an urgent priority. Efforts to develop HIV-1 vaccines are hampered by the absence of models of naturally occurring protective immunity to HIV-1. Characterization of immune responses to HIV-1 that protect against infection would help to provide a focus for HIV-1 vaccine research and could speed the development of effective vaccines.

With the recognition of the type 1/type 2 dichotomy in the T-helper cell response to infection,2,3 the concept that cellular effector mechanisms may mediate protective immunity to HIV-1, in the absence of systemic humoral immunity, has gained credence over the past several years.4,5 Several groups of HIV-1 exposed uninfected and seronegative individuals with evidence of T-cell responses to HIV-1 antigens have been reported.6-9 Clerici et al. have described a group of gay men who developed T-cell responses to HIV-1 after several unprotected sexual exposures to HIV-1 infected partners.7 MHC Class I-restricted CTL to HIV-1 have been reported in uninfected children of HIV-1-infected mothers,8-10 exposed health-care workers,11 uninfected heterosexual partners of HIV-1-infected individuals12 and HIV-1- and HIV-2-uninfected prostitutes in the Gambia.13 These findings have led to the suggestion that cellular rather than systemic humoral immunity may be important in protection against HIV-1 infection.14-16 According to this hypothesis, a systemic humoral or type 2 T helper response represents an ineffectual immune response, whereas the generation of a cellular, type 1 immune response is effective in eliminating the virus and preventing the establishment of detectable infection. Evidence for the ability of the cellular immune response to control virus levels comes from the recent observation that, among long-term non-progressors, HIV-1-specific T helper responses are inversely correlated with viral load.17 The detection of HIV-1-specific cellular immune responses in HIV-1-exposed but uninfected individuals suggests that these individuals have had exposures to HIV-1 sufficient to induce
cellular immune responses but insufficient to induce antibody. This could result from exposure to defective virus or HIV-1 antigens. A second possibility is that these individuals have been infected with HIV-1 and cellular immune responses have been responsible for clearance of infection, as has been documented in children born to HIV-1-infected mothers. However, the studies reported to date do not provide evidence that these responses protect on subsequent exposure. Rather, they only suggest that some individuals are able to clear an HIV-1 infection. To examine whether immune responses can contribute to protection against infection, a study would be required in which these responses are detected in a setting where high levels of exposure to HIV-1 provide sufficient opportunity for HIV-1 infection, and yet there is evidence that the individuals are not infected.

There is evidence for protection against HIV-1 infection from observational epidemiological studies. Early studies of risk factors for sexually acquired HIV-1 infection have suggested that the risk of becoming infected is independent of exposure. Among Nairobi prostitutes, the mean duration of prostitution is considerably shorter in HIV-1-infected women compared to uninfected women. Similarly, in the sex partners of HIV-1-infected haemophiliacs and the sexual partners of HIV-1-resistant seronegative women to participate in this particular arm of the study. 21 One or more occasions (Novapath Immunoblot, BioRad, Hercules, CA, USA).

Methods for HIV-1 PCR have been previously described. In brief, conserved regions of the env, vif and nef genes were targeted for amplification. Because two of the three primer sets are in regulatory genes, which are not involved in defining HIV-1 clades, this amplification strategy is capable of detecting HIV-1 variants from multiple clades, including those most prevalent in East Africa. All women classified as resistant had tested negative for HIV-1 PCR on one or more occasions.

In vitro studies

Blood was drawn by venipuncture into sodium heparin vacutainers (Becton Dickinson, Mississauga, Ontario, Canada) or into citrate, phosphate, dextrose and adenine (CPDA) blood collection bags (Red Cross Society International, Nairobi, Kenya). Peripheral blood mononuclear cells were purified by Ficoll-Hypaque (Sigma, St Louis, MO, USA) density gradient centrifugation. All cell cultures were performed in RPMI-1640 supplemented with 10 mmol/L HEPES and 1-glutamine pH 7.2 (Gibco BRL, Gaithersburg, MD, USA), 50 mmol/L β-mercaptoethanol (Sigma), 10 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 mg/mL amphotericin B and 10% fetal calf serum (Intermed, Intermed, Issaquah, WA, USA), which had been heat inactivated at 56°C for 40 min.

T helper assay

Interleukin-2 and IL-6 production in response to different stimuli were used to assay T helper cell function. The methods for detection of IL-2 production by PBMC in response to HIV-1 and control antigens were based on those of Clerici et al. 7 Briefly, PBMC were isolated and cryopreserved until all samples could be assayed simultaneously. In a 96-well plate, 3 × 10^5 PBMC were incubated with test solutions (triplicate wells per test solution) in the presence of 25 µL of a 1:100 dilution of anti-Tac monoclonal antibody (clone 322a, specific for the alpha chain of the IL-2 receptor, was provided by the Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA). Test solutions used were media, recombinant soluble gp120 (Intracel Corporation, Issaquah, WA, USA) 2 µg/mL, each of five different HIV-1 env peptides (AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH, Bethesda, MD, USA) 2.5 µg/mL and

Materials and Methods

Study subjects

Female sex workers enrolled in the Pumwani Sex Worker Cohort in Nairobi, Kenya were the source of HIV-1-resistant and HIV-1-infected subjects for the studies described here. All participants in this study, including controls, gave informed consent and this study conformed to all ethical guidelines from the University of Manitoba and the University of Nairobi. Procedures for enrolment and follow up and the HIV-1 and sexually transmitted disease (STD) interventions offered through the clinic have been previously described. 19,23 Studies of the epidemiology, immunobiology and prevention of sexually transmitted infections are nested within the cohort for three or more years, were HIV-1/2 seronegative and had normal CD4+ counts. Women meeting the epidemiological definition of resistance to HIV-1 infection were invited to participate in this study of mechanisms of resistance to HIV-1 infection and gave their informed consent. Due to the fact that many of the women do not permanently reside in Nairobi, we were only able to contact and recruit 24 of the 80 resistant women to participate in this particular arm of the study. The HIV-1-seropositive women from the Pumwani Sex Worker Cohort were also approached about their participation. The HIV-1 seronegative controls, who were at low risk of HIV-1 exposure, were selected from Kenyan women enrolled as negative controls in a study of mother-to-child transmission of HIV-1. In the latter subjects, the last pregnancy was more than 1 year prior to participation in this study. Additional low-risk seronegative controls included Kenyan laboratory workers and some North American laboratory workers (all of whom had resided in Nairobi for 6 months or more).

Detection of HIV-1

All individuals in the study were tested for the presence of HIV-1/2 antibodies with commercial enzyme immunoassays (HTLV-III ELISA, DuPont, Wilmington, PE, USA from 1985 to 1988; Vironostika, Organon Technika, Durham, NC, USA from 1988 to 1990; Detect HIV, IAF Biochem, Laval, Quebec, Canada from 1990 to 1992; and Enzygnost HIV-1/2 EIA, Behring, Marburg, Germany from 1991 to present). These serology systems are capable of detecting infection resulting from all of the HIV-1 clades predominant in East Africa and HIV-2, which has not been reported in that region. 21,26 All women defined as resistant also had HIV-1 immunoblots performed on one or more occasions (Novapath Immunoblot, BioRad, Hercules, CA, USA).

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phytohaemagglutinin-P (PHA-P, Sigma) 5 µg/mL as a positive control. These peptides were chosen because they have been previously shown to elicit IL-2 responses in HIV-infected and exposed uninfected individuals.\textsuperscript{7,28} In these experiments, the media control also served as an irrelevant antigen control, because the fetal calf serum in the media contains many non-HIV-1 proteins and peptides. Peptides have been listed by NIH catalogue number with the corresponding peptide name used in the previous studies\textsuperscript{7,28} in parentheses and the amino acid location within HIV-1\textsubscript{env}, gp120 (env): 1929 (T2) env 101–120, 864 (P18) env 306–327, 1991 (P23) env 361–380, 2007 (T1) env 421–440 and 1589 (T1) env 418–441. Samples were incubated at 37°C for 7 days and the supernatants were collected and frozen at –70°C and shipped on dry ice to the University of Manitoba where they were thawed and assayed. These same culture supernatants were used for IL-6 determinations. Production of IL-2 and IL-6 was measured using commercial enzyme immunoassays performed according to the manufacturer’s instructions (Quantikine Immunoassays, R & D Systems, Minneapolis, MN, USA). The PBMC from all groups (resistant, HIV infected and low risk) were handled similarly and assayed on the same plates.

**Cytotoxic T cell assay**

Cytotoxic T cell assays were performed as described by Grant et al.\textsuperscript{29} Both fresh (assayed in Nairobi) and cryopreserved cells (assayed at the University of Manitoba) were used in these assays and the results proved reproducible. Briefly, 3-day-old PHA blasts were infected with HIV-1\textsubscript{inu} inactivated with mitomycin C and used as autologous stimulators for bulk autologous PBMC (effectors), which were cultured for 7 days with recombinant human IL-2 (5 U/mL, Roche Diagnostics, Laval, Quebec, Canada). On day 10 of the assay, autologous EBV-transformed B cells (targets) were infected with one of four recombinant vaccinia/HIV-1 viral vectors. The recombinant vaccinia/HIV-1 viruses included vSC8 (wild-type vaccinia with the β-galactosidase gene),\textsuperscript{30} vDK1 (HIV-1\textsuperscript{gag} inserted into vSC8), vCF21 (HIV-1\textsuperscript{pol} inserted into vSC8)\textsuperscript{31} and vPE16 (HIV-1\textsuperscript{env} inserted into vSC8).\textsuperscript{32} Viral vectors were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH. The following day, [\textsuperscript{51}Cr]-labelled target cells were washed and plated in triplicate, with the effectors, at effector to target ratios of 50:1, 25:1 and 12.5:1. Wells containing only target cells or target cells with 1% SDS were used to determine the spontaneous (Min) and maximal release (Max), respectively, of \textsuperscript{51}Cr from the labelled cells. After 4 h incubation, 100 µL of supernatant were removed and added to 1 mL of scintillation cocktail (Ecolume, ICN, Costa Mesa, CA, USA) and the released \textsuperscript{51}Cr detected using a liquid scintillation counter. Percent spontaneous release was calculated as Min/Max × 100. Percent specific lysis was calculated by ((Exp – Min)/(Max – Min)) × 100. Lysis of the targets infected with the recombinant vaccinia/HIV-1 (vDK1, vCF21 and vPE16) viruses was considered HIV-1 specific if lysis was 10% or greater than the lysis of the vaccinia control (vSC8) and the results were titratable or were repeatable.

CD8\textsuperscript{+} lymphocyte-depleted cultures were prepared by a 30 min exposure of the PBMC cultures to immunomagnetic beads specific for the CD8\textsuperscript{+} molecule (Dynal, Inc., Lake Success, NY, USA) and elution of the CD8\textsuperscript{+} T cell-depleted PBMC for use in CTL assays.

**Data analysis**

Data were analysed using the Statistical Package for the Social Sciences (SPSS) version 7.1 microcomputer program. Chi-squared test and Student’s \( t \)-test were used for comparison of sample proportions and means. Linear discriminant analysis\textsuperscript{33} was used to analyse the relationship between T-helper responses in the resistant and control groups. Student’s \( t \)-tests were used to evaluate the relationship between T-helper and CTL responses. Discriminant analysis discriminates two populations by a linear set of variables (for instance IL-2 secretion at baseline and IL-2 secretion in response to peptide stimulation). The distributions of these variables in the two populations are assumed to be normal and have the same (co)variance structure. It is similar to logistic regression, but more efficient for small samples. The conclusions drawn from discriminant analysis are not dependent on asymptotic approximations (which may be most in small samples such as ours). Discriminating variables were logarithmically transformed in order to make their distributions approximate to the above assumptions.

**Results**

**Infection status of the study subjects**

Twenty-four women who were classified as resistant to HIV-1 were available to participate in these studies. All were HIV-1 antibody negative by both enzyme immunoassay and immunoblot. Negative serology on each individual was performed 8–56 times (mean ± SD, 21 ± 13) over 4–10 years. All women were HIV-1 PCR negative on specimens obtained simultaneously with the studies reported here. None of the HIV-1-resistant women participating in the present study have subsequently seroconverted to HIV-1 in an additional 24 months of follow up, despite continued exposure to HIV-1 through sex work.

The HIV-1 systemic antibody status of the positive and negative control groups was confirmed by enzyme immunoassay.

**CD4 Levels**

CD4 levels were not available for the same day that the test subjects were bled for this study. However, as an estimate we took the CD4 values for time points that were < 3 months earlier and later that this study date. A Student \( t \)-test analysis showed no difference between the earlier and later values. The average of the two points was taken for each individual and means generated for each group (low risk 758 CD4/mm\textsuperscript{3}, resistant 1019 CD4/mm\textsuperscript{3} and HIV-infected 434 CD4/mm\textsuperscript{3}). Student’s \( t \)-test analysis showed that while the CD4 values for the low-risk and resistant groups did not significantly differ (\( P = 0.16 \)), both had significantly higher CD4 levels than the HIV-infected group (\( P = 0.04 \) and \( P = 0.001 \), respectively).

**HIV-1-specific T-helper responses**

To determine whether the resistant women exhibit HIV-1 specific immune responses, PBMC from 17 resistant, seven HIV-1-infected and six low-risk seronegative subjects (five Kenyan women and one North American laboratory worker) were cultured in the presence of sgp120 and each of the HIV-1 envelope peptides separately. After 7 days of culture, the supernatants were assayed for IL-2 levels by enzyme immunoassay. Table 1 and Fig. 1 show the median IL-2 secretion. The HIV-1-infected group exhibited spontaneous IL-2 secretion. Twelve of 17 resistant women, no low-risk seronegative subjects and no HIV-1-infected women showed
Table 1  Interleukin-2 secretion following stimulation of PBMC with media and HIV-1 antigens

<table>
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<th>Study Number</th>
<th>Status</th>
<th>Media</th>
<th>sgp120</th>
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<th>p23</th>
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<td>9 (1)</td>
<td>9 (0)</td>
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<td>6 (0)</td>
<td>10 (4)</td>
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<td>6 (0)</td>
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<td>21 (13)</td>
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<tr>
<td>Median</td>
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<td>6.5</td>
<td>6.0</td>
<td>6.0</td>
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</tr>
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</table>

ML 466 Resistant 7 (1) 16 (2) 7 (1) 6 (0) 10.5 (5) 6 (0) 8 (0)
ML 857 Resistant 90 (22) 104 (31) 95 (23) 68 (12) 92 (24) 65 (22) 106 (31)
ML 858 Resistant 112 (39) 70 (3) 71 (4) 51 (11) 124 (26) 51 (8) 81 (1)
ML 887 Resistant 15 (5) 12 (6) 8 (2) 14 (9) 8 (3) 11 (4) 10 (2)
ML 889 Resistant 34 (7) 64 (10) 56 (9) 31 (7) 35 (2) 31 (8) 25 (1)
ML 893 Resistant 129 (40) 159 (17) 73 (4) 97 (41) 93 (29) 77 (23) 175 (53)
ML 935 Resistant 34 (12) 46 (2) 47 (3) 49 (14) 50 (5) 53 (6) 75 (21)
ML 1025 Resistant 36 (9) 53 (37) 36 (12) 41 (6) 24 (19) 43 (19) 14 (5)
ML 1250 Resistant 32 (20) 47 (5) 24 (2) 45 (3) 38 (12) 37 (9) 47 (2)
ML 1260 Resistant 6 (0) 8 (2) – 6 (1) – 6 (1) 9 (4)
ML 1275 Resistant 18 (2) 46 (12) 42 (8) 37 (4) 36 (11) 37 (14) 36 (2)
ML 1356 Resistant 7 (1) 14 (3) 6 (0) 6 (0) 13 (6) 17 (8) 9 (4)
ML 1358 Resistant 12 (6) – 31 (2) 43 (2) 16 (11) 13 (7) 14 (9)
ML 1362 Resistant 19 (8) 15 (1) 16 (8) 11 (5) 9 (4) 15 (1) 11 (2)
ML 1371 Resistant 9 (3) 10 (4) 8 (2) 15 (8) 8 (3) 8 (3) 17 (8)
ML 1376 Resistant 64 (6) 196 (18) 245 (7) 182 (8) 149 (20) 108 (20) 188 (36)
ML 1490 Resistant 21 (9) 23 (1) 30 (1) 31 (3) 27 (5) 22 (12) 25 (1)
ML Median    | 21.0     | 46.0  | 31.0  | 37.0    | 30.0    | 37.0 | 25.0 |

ML 566 HIV+ 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0)
ML 1018 HIV+ 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0)
ML 1075 HIV+ 8 (3) 7 (2) 6 (0) 8 (2) 7 (1) 8 (3) 8 (3)
ML 1359 HIV+ 6 (0) 6 (0) 6 (0) 8 (2) 7 (1) 6 (0) 6 (0)
ML 1382 HIV+ 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0)
ML 1563 HIV+ 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0)
ML 1728 HIV+ 9 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0) 6 (0)
ML Median 6.0 6.0 6.0 6.0 6.0 6.0 6.0

P value, Low risk versus resistant 0.007 0.003 0.004 0.001 0.004 0.007 0.010
P value, HIV+ versus resistant 0.002 < 0.001 0.001 0.001 0.001 0.002 0.002
P value, discriminant analysis† – < 0.001 < 0.001 < 0.001 < 0.001 < 0.001

For HIV-1 resistant women (resistant), low risk controls (low risk) and HIV-1-infected sex workers (HIV+), mean IL-2 secretion in pg/mL (with SEM in parentheses) is shown for each stimulus. The median IL-2 production for each group is listed. The limit of IL-2 detection was 6 pg/mL. *Two-tailed Student’s t-test, †baseline media responses were entered into the discriminant model for each antigen and were < 0.001 in each instance. Bold type indicates ≥ twice IL-2 production of media alone. ML, samples derived from the Pumwani Sex Worker Cohort; MCH, samples are HIV negative controls from the mother-to-child HIV-1 transmission study; NA, HIV negative North American control.

spontaneous IL-2 responses of greater than threefold the lower limit of detection in the assay with the media control (Chi-squared test, P < 0.001). Interleukin-2 responses to all stimuli were significantly greater in the HIV-1-resistant group compared with both HIV-1-infected and low-risk controls. However, because of the significantly higher baseline responses in the resistant group we performed a discriminant analysis of the responses to HIV-1 antigens, controlling for the potential confounding effects of IL-2 responses to media, by including it as an additional discriminating variable. This confirmed that, after controlling for IL-2 responses to media, resistant women have statistically significant higher responses to the HIV-1 antigens (t-test P < 0.01 for each peptide and sgp120). As another way of controlling for baseline responses, we calculated stimulation indices for responses to the HIV-1 peptides and sgp120. Among the groups who have studied T-helper responses in HIV-infected and exposed seronegatives,7,11,17,28,34–36 various arbitrary stimulation index cut-off values have been selected. Seven of the 17 (41%) resistant women tested had stimulation indices (SI) of ≥ 2.0 against the HIV-1 antigens. Four of those seven responded to two or more HIV-1 antigens. One of the six low-risk negative controls and one of the seven HIV-1-positive subjects had a single HIV-1 antigen that had an SI of ≥ 2.0. When an SI of ≥ 2.0 was used to define responders, there were 0/6 low risk, 0/7 HIV-1 positive and 4/17 resistant women who responded, two of whom did so to two or more antigens. Three of the 17 resistant women had SI of ≥ 3.0 with one responding to two or more antigens. To better illustrate the trends in responses to the various HIV-1 antigens, a
summary of the IL-2 experiments is presented in Fig. 1. The IL-2 production (pg/mL) in response to stimulation by the HIV-1 peptides and soluble gp120 for the resistant women is significantly greater than either the low-risk (t-test, P < 0.01) or HIV-1-infected individuals (t-test, P < 0.002). Interleukin-6 production at baseline and in response to HIV-1 peptides and PHA was not different between the three study groups (data not shown).

Cytotoxic T cells to HIV-1 antigens
Twenty-two HIV-1-resistant prostitutes, eight low-risk Kenyan women and four laboratory workers were tested, some on multiple occasions, for HIV-1-specific CTL. As shown in Fig. 2a, 15 of 22 resistant women showed levels of env-specific lysis that were equal to or greater than 10% above that seen in targets infected with vaccinia alone. Of nine women tested with the vaccinia-gag construct, three were positive (Fig. 2b). Two of seven women tested had ≥ 10% specific lysis of the vaccinia-pol target (Fig. 2b). All 22 women were tested for env responses and of the nine women who had sufficient numbers of effector cells to test for gag and/or pol responses, four tested positive to more than one HIV-1 antigen, with subject 1376 being positive to all three targets. Overall, 15 of 22 resistant prostitutes showed significant levels of CTL to one or more of the vaccinia/HIV-1 vectors. The HIV-1 specific cytotoxic activity was not detected in seven of the resistant prostitutes. None of the 12 HIV-1 seronegative low-risk test subjects showed significant specific lysis of the vaccinia/HIV-1 constructs, which is significantly fewer responders than the resistant women (0/12 versus 15/22; Chi-squared test, P < 0.001). The depletion of CD8+ lymphocytes from the effector PBMC by immunomagnetic beads, performed on two subjects, reduced the high levels of cytotoxic activity achieved with the vaccinia/env construct to that of the vaccinia construct alone (Fig. 2c). A comparison of various attributes of women with and without HIV-1-specific CTL responses showed no differences between the two groups. Values for CTL+ (n = 15) and CTL− subjects (n = 7) and P values, respectively, for various parameters were as follows: age, 37.7 years, 36.1 years, 0.6; CD4/mm3, 970, 1088, 0.6; CD8/mm3 732, 863, 0.4; CD4/CD8 ratio 1.4, 1.3, 0.3; clients/day 3.9, 5.0, 0.3; condom use 85.5%, 79.1%, 0.6; duration of commercial sex work (years) 10.7, 10.2, 0.9.

Comparison of tested versus non-tested resistant women
The 24 resistant women who participated in this immunological study were unselected and did not differ significantly (by Student’s t-test) from the 56 untested resistant women in terms of age, proportion of visits with gonorrhea, episodes of genital ulcers, number of sex partners per day and duration of prostitution prior to cohort entry (Table 2). Studied women had a longer duration of follow up and, because condom use had increased over the period of observation, reported a slightly greater average condom use (over the period of observation). The women tested were also similar to those not tested for HLA class I alleles, which are associated with an increased or decreased risk of HIV-1 seroconversion in epidemiological studies.37 Although only 24 women were available at the time of the present study, the other 56 women have subsequently been seen in the clinic and remain HIV uninfected despite continued high-risk activity.

Association between cytotoxic T cells and T-helper responses
Cytotoxic T lymphocyte assays and T helper cell assays were both performed on 15 resistant women. Eleven of these women had significant CTL to one or more HIV-1 antigens. Student’s t-test confirmed an association between the presence of CTL and IL-2 secretion to the env peptides T1-1589 (P = 0.035), T1-2007 (P = 0.051), T2 (P = 0.024), p18 (P = 0.049), p23 (P = 0.001) and sgp120 (P = 0.014).
Figure 2 (a) Cytotoxic T-cell responses among 22 resistant prostitutes to autologous B lymphoblastoid targets infected with vaccinia/env constructs. The data are plotted as percentage specific lysis, which is defined as the lysis of targets infected with the control vaccinia subtracted from the lysis of vaccinia/env-infected targets. Three effector to target (E:T) ratios were used (12.5:1, 25:1 and 50:1), although for clarity only 50:1 is shown, except for patient 1490 in which the 25:1 dilution is shown. (b) Cytotoxic T cell responses to autologous B lymphoblastoid targets infected with vaccinia/gag and vaccinia/pol constructs. The data are plotted as percentage specific lysis. Three E:T ratios were used: 12.5:1 (□), 25:1 (□) and 50:1 (■). (c) Cytotoxic T cell responses of two resistant women to autologous B lymphoblastoid targets infected with vaccinia and vaccinia/env constructs following CD8+ depletion of effector cells. The percentage lysis is plotted. Three E:T ratios were used: 12.5:1 (□), 25:1 (□) and 50:1 (■). VAC, targets infected with vaccinia; VAC ENV, targets infected with vaccinia/env; –CD8, CD8-depleted PBMC used as effectors.
HIV-1 for their cognate receptors, could mediate resistance or potentially be involved in resistance. It has been suggested that soluble factors that inhibit HIV-1 replication could potentially mediate resistance. We have investigated a number of these potential mechanisms in the Nairobi sex workers. We have determined that altered in vitro cellular susceptibility to T-cell- and macrophage-tropic HIV-1 infection, CCR5 and CCR3 polymorphisms, altered expression of CCR5 and CXCR4 proteins and overproduction of β-chemokines do not explain the resistance phenomenon. Soluble factors produced by CD8" T cells that inhibit HIV-1 replication but are distinct from chemokines are also found in individuals who are exposed to HIV-1 but escape infection. We are currently investigating the role of these CD8" soluble factors in the Nairobi cohort.

Cellular immune responses (CTL and T helper cell) have been implicated in the control of viral infections. It is important to define immune mechanisms that may mediate resistance to HIV-1 infection and provide a basis for the design of effective vaccines for HIV-1 containment. The importance of HIV-1-specific T-helper responses in controlling the HIV-1 viraemia has been re-emphasized by Rosenberg et al. They have observed that, among long-term non-progressors and patients on protease inhibitor therapy, HIV-1-specific T-helper responses correlate with low viral titres. In the present study, resistant women exhibited significantly greater production of IL-2 in response to HIV-1 antigens than either low-risk seronegative controls or infected sex workers, suggesting that they have T helper cell memory for HIV-1 antigens (Table 1; \( P < 0.01 \), Student’s t-test). An important observation is that the resistant women had statistically higher IL-2 baseline responses \( (P < 0.01, \) Student’s t-test) than the low-risk controls (Table 1). The reason for this is not known. Because experiments in the three study groups were performed simultaneously and under identical conditions, it seems unlikely that this is an experimental artefact. Furthermore, because the spontaneous IL-6 secretion was not statistically higher in resistant women, this appears to be a phenomenon that is specific to IL-2. One possibility is that these high IL-2 responses are essential to the resistant state and are involved in protection against HIV-1 infection. Alternatively, these responses may result from a highly activated immune state associated with protection against HIV infection. Further studies of cellular activation markers in conjunction with cytokine production in this group of women are planned and would help to clarify these possibilities.

Despite the higher spontaneous IL-2 levels, superimposed HIV-specific responses were also detected among the resistant women but not in controls. We used two methods of analysis to control for the higher IL-2 baselines. First, IL-2 responses were compared by discriminant analysis in the three groups, controlling for baseline IL-2 responses. For each test peptide, IL-2 responses were significantly greater in resistant women than controls (Table 1; \( P < 0.001, \) discriminant analysis). We also compared all test peptide IL-2 responses relative to the unstimulated control, that is, the stimulation index (SI), although this is a less than ideal statistical technique. In previous studies of T-helper responses among HIV-1-infected or exposed seronegatives, an arbitrary

### Table 2 Comparison of the HIV-resistant women who participated in this immunological study and those resistant women who did not

<table>
<thead>
<tr>
<th>Category of comparison</th>
<th>Not tested ± SD (( n = 56 ))</th>
<th>Tested ± SD (( n = 24 ))</th>
<th>( P ) value (2 tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.90 ± 6.00</td>
<td>31.10 ± 5.30</td>
<td>0.580</td>
</tr>
<tr>
<td>Condom use</td>
<td>2.7 ± 0.9</td>
<td>3.20 ± 0.50</td>
<td>0.013</td>
</tr>
<tr>
<td>Duration of follow up (years)</td>
<td>4.50 ± 1.80</td>
<td>6.00 ± 2.00</td>
<td>0.001</td>
</tr>
<tr>
<td>Proportion of visits with gonorrhea</td>
<td>0.35 ± 0.28</td>
<td>0.24 ± 0.13</td>
<td>0.070</td>
</tr>
<tr>
<td>Episodes of genital ulcers</td>
<td>4.10 ± 7.50</td>
<td>6.20 ± 5.80</td>
<td>0.225</td>
</tr>
<tr>
<td>Sex partners per day</td>
<td>3.60 ± 2.20</td>
<td>3.40 ± 2.10</td>
<td>0.700</td>
</tr>
<tr>
<td>Duration of prostitution prior to entry (years)</td>
<td>4.90 ± 4.40</td>
<td>5.40 ± 6.60</td>
<td>0.690</td>
</tr>
</tbody>
</table>

### Discussion

Over the past several years, evidence for heterogeneity in susceptibility to HIV-1 infection has accumulated. Some of the strongest epidemiological evidence for resistance to HIV-1 infection comes from observational studies of women in the Pumwani Sex Worker Cohort in Nairobi. These women have intense exposure to HIV-1 through their occupation and, although condom use is frequent (> 80% of sexual encounters), their risk of acquiring HIV-1 infection is enormous. Despite this intense exposure of up to 500 unprotected sexual exposures to HIV-1-infected clients, a small number (13% of initially HIV-1 seronegative women) remain HIV-1 uninfected for prolonged periods (up to 13 years). The risk of HIV-1 infection has been shown to be heterogeneous and to decline with time. Women who remain HIV-1 uninfected after 3 years are at a substantially reduced risk of subsequent HIV-1 infection. In previous studies, we have shown that this plateau in HIV incidence is not a chance phenomenon and is not related to safer sexual behaviour, altered susceptibility to factors increasing the risk of HIV-1 infection or seronegative HIV-1 infection. Having excluded these other possibilities, we have concluded that, on an epidemiological basis, these women are resistant to HIV-1. The present study shows that a high proportion of resistant women from the Pumwani Sex Worker Cohort have HIV-1-specific cellular immune responses.

Several mechanisms of resistance to HIV-1 infection are beginning to be understood. Polymorphisms in chemokine receptors, which serve as HIV-1 coreceptors, mediate cellular resistance to infection. As an example, cellular resistance to macrophage-tropic HIV-1 isolates is associated with a polymorphism in the CCR5 receptor (Δ32CCR5), which results in the absence of its expression. The heterozygous condition is found at a frequency of approximately 11% in the Caucasian population, but not at all among 261 native Africans (many of whom were Kenyan) studied. Several soluble factors that inhibit HIV-1 replication could potentially be involved in resistance. It has been suggested that increased production of β-chemokines, which compete with HIV-1 for their cognate receptors, could mediate resistance. We have investigated a number of these potential mechanisms in the Nairobi sex workers. We have determined that altered in vitro cellular susceptibility to T-cell- and macrophage-tropic HIV-1 infection, CCR5 and CCR3 polymorphisms, altered expression of CCR5 and CXCR4 proteins and overproduction of β-chemokines do not explain the resistance phenomenon. Soluble factors produced by CD8" T cells that inhibit HIV-1 replication but are distinct from chemokines are also found in individuals who are exposed to HIV-1 but escape infection. We are currently investigating the role of these CD8" soluble factors in the Nairobi cohort.

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range of stimulation index cut-off values have been used,\textsuperscript{7,11,17,28,34-36,52} Kelker et al. have compared proliferative responses in HIV-infected individuals and their exposed seronegative partners (who had been exposed a median of 600 times) using SI of 2.0, 2.5 and 3.0 and performed comparisons of multiple antigen responses using the 2.0 cut-off.\textsuperscript{36} Using an SI cut-off of 2.0,\textsuperscript{7,28,35,36} we have shown that 1/6 (17%) low risk seronegatives, 1/7 (14%) HIV positives and 7/17 (41%) resistant women responded to the HIV antigens. Four of those seven responded to two or more antigens. Using higher SI cut-offs of 2.5 and 3.0 eliminates the positive responses among the low-risk seronegatives and HIV positives and reduces the number of responders among the resistant women to 4/17 (24%) and 3/17 (18%), respectively. The absence of HIV-1-specific T-helper responses in the HIV-1 infected group is not surprising, considering their advanced immune dysfunction as indicated by their significantly lower CD4 cell levels. Using an SI of $\geq$ 2.0, Kelker et al. have found that, among the exposed seronegatives, 60% responded to one or more antigens and 43% responded to two or more antigens.\textsuperscript{36} It is worth noting that other studies have used fresh cells, while we used cryopreserved PBMC. Because it is known that freezing affects the ability of APC to efficiently present antigens, it is highly probable that the use of cryopreserved PBMC contributed to our more modest responses. In addition, our responses may have been lower because other studies have used whole-protein antigens,\textsuperscript{17} which possess multiple epitopes, whereas we used primarily single-epitope HIV peptides. It is also likely that the MHC class II allelic frequencies of our study population are significantly different from previously reported groups\textsuperscript{17,28,36} and therefore could represent differences in peptide presentation rather than strength of responses. Therefore, it is not unexpected that the level of immune response is lower than that observed by others. Significantly, the responses detected in the present study were to clade B peptides, while the population has been primarily exposed to clades A, C and D viruses.\textsuperscript{25,26} This suggests that either these epitopes are conserved or that there is significant cross-reactivity of these epitopes across clades. This is supported by the recent observation of cross-clade CTL among HIV-1-infected individuals\textsuperscript{35} and by other studies in this cohort,\textsuperscript{34} in which resistant women have been shown to have stronger CTL responses to A and D clade epitopes than to B clade epitopes.

The specificity of the T-helper responses is suggested by the observation that among the seven resistant women who had SI $\geq$ 2.0 to the HIV-1 antigens, the pattern of HIV-1 antigens that they responded to was very different for each individual. This suggests that the responses are not due to a non-specific increase in IL-2 production to every peptide, but rather that each subject specifically responds to a different set of antigens (Table 1). The present study is the first to demonstrate T-helper responses among seronegative highly exposed sex workers who are epidemiologically resistant to HIV-1 infection.

Several groups have reported HIV-1-specific CTL in individuals who are exposed but not infected with HIV-1. MHC class I-restricted CTL to HIV-1 have been reported in the uninfected children of HIV-1-infected mothers,\textsuperscript{5,9} uninfected regular heterosexual partners of HIV-1-infected individuals,\textsuperscript{12} health-care workers with occupational exposure to HIV-1\textsuperscript{11} and HIV-1-uninfected prostitutes in the Gambia.\textsuperscript{13} The women who constituted our study population have had high HIV exposure, with up to 500 unprotected sexual exposures to HIV-1-infected men over the period of up to 13 years.\textsuperscript{22} Mathematical modelling has shown that statistically these women should be infected if all women were equally susceptible.\textsuperscript{22} Overall, we show that CTL to HIV-1 targets are detectable in a high proportion (68%) of resistant women tested. We found CTL activity mainly against env constructs, but also some against gag and pol. The CD8+ cell depletion experiments (Fig. 2c) suggest that, for those two individuals tested, the cytotoxic activity observed in these CTL assays was mediated by CD8+ T cells.

The present study is among the few\textsuperscript{31,52} that have reported HIV-1-specific T-helper and CTL responses among highly exposed seronegative individuals. In the present study, among those individuals to whom both assays were performed, CTL activity was significantly correlated (by Student’s $t$-test) with all of the HIV-1 peptides used in the T-helper assays. It is possible that the IL-2 provided through the significantly higher spontaneous and HIV-1-specific helper responses could be important in the development and maintenance of HIV-1-specific CTL. This suggests that it may be important for future HIV-1 vaccines to incorporate both T-helper and CTL epitopes into vaccines and to monitor those responses.\textsuperscript{55} Resistant women who do not have detectable HIV-1-specific cellular responses may have CTL and T helper cells that recognize HIV-1 antigens not tested in these studies or may be protected by mechanisms other than systemic HIV-1 cellular immune responses, such as mucosal immune responses.\textsuperscript{56-59}

In conclusion, we have conducted a number of studies to attempt to determine potential mechanisms responsible for HIV-1 resistance observed among some of the women of the Nairobi prostitute cohort.\textsuperscript{64} Many of the HIV-1-resistant women tested exhibit evidence of HIV-1-specific T helper and CTL responses. These findings support the concept that cellular immune responses are important and may contribute to providing protection against HIV-1 infection and should be a central consideration when developing HIV-1 vaccine strategies.

Acknowledgements

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References

et al.

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