Persistently HIV-1 seronegative Nairobi sex workers are susceptible to in vitro infection

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OBJECTIVE: To evaluate whether resistance to HIV-1 infection in a subset of highly exposed sex workers correlates with resistance at the cellular level.

DESIGN: In vitro evaluation of susceptibility to infection by Kenyan HIV-1 isolates and cellular production of potential mediators of resistance.

SETTING: Samples were collected in a primary care clinic in Nairobi.

PATIENTS: Thirteen individuals from a cohort of sex workers with a similar risk of acquiring HIV infection and six unexposed controls.

INTERVENTIONS: Subjects were provided with appropriate primary care and counselling on the prevention of sexually transmitted diseases.

RESULTS: No inherent cellular resistance to infection was identified. CD8⁺ cells from a subset of subjects strongly inhibited viral replication.

CONCLUSIONS: Lack of infection in this cohort was not attributable to factors inherent to CD4⁺ cells. Resistance to HIV infection is likely to be multifactorial, and products of CD8⁺ cells and unique features of mucosal sites probably contribute to this state.

Key Words: CD8 cell-mediated suppression; HIV; HIV resistance; Sex workers

Des travailleuses du sexe de Nairobi demeurés VIH-négatifs sont sensibles à l'infection *in vitro*

OBJECTIF : Vérifier si la résistance à l'infection au VIH chez une sous-population de travailleuses du sexe de Nairobi fortement exposée est en corrélation avec la résistance à l'échelon cellulaire.

MODÈLE : Évaluation *in vitro* de la sensibilité à l'infection par des isolats de VIH-1 kényans et production cellulaire de modulateurs potentiels de la résistance.

CONTEXTE : Des échantillons ont été prélevés dans une clinique ambulatoire de Nairobi.

PATIENTS : Treize personnes d'une cohorte de travailleuses du sexe exposés au même risque de contamination par le VIH et six témoins non exposés.

INTERVENTIONS : Les sujets ont reçu le counselling approprié sur la prévention des maladies transmissibles sexuellement et sur les soins de base.

RÉSULTATS : Aucune résistance cellulaire inhérente contre l'infection n'a été identifiée. Les cellules CD8⁺ d'une sous-série de sujets ont fortement inhibé la réplication virale.

CONCLUSIONS : L'absence d'infection dans cette cohorte n'a pas été jugée attribuable à des facteurs propres aux cellules CD4⁺. La résistance contre l'infection est probablement plurifactorielle et les produits des CD8⁺ et des caractéristiques uniques des sites muqueux contribuent probablement à ce phénomène.

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Several studies have reported on individuals that remain persistently seronegative despite repeated exposure to the HIV type 1 (HIV-1). These have included health care workers with accidental exposure (1), infants born to infected mothers (2-4), needle-sharing intravenous drug users (5), individuals engaged in unprotected sexual intercourse (6,7) and prostitutes (8,9). The risk for infection among these cohorts varied greatly, and T cell-mediated immunity, as measured by interleukin (IL)-2 production, lymphocyte proliferation in response to HIV-derived peptides or HIV-specific cytotoxic T lymphocytes, was thought to contribute to resistance in these cases (1-4,6,7,9,10). The proposed mechanism involved priming of T cell responses with low antigenic doses, and generation of cytokine-mediated T helper cell type 1 (Th1) immune responses, which upregulate cellular effector functions and downregulate T cell help for B cells (11,12).

The beta-chemokines macrophage inflammatory protein (MIP)-1-alpha, MIP-1-beta and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) are produced by CD8⁺ cells, inhibit viral replication (13) and bind to the main HIV coreceptor CCR5 (14,15). Homozygous mutation of CCR5 imparted marked (15) but incomplete (16-18) resistance. Inherent cellular resistance was attributed to overproduction of beta-chemokines (19), stimulation of CD3/CD28 pathways (20,21), and suppression of HIV transcription by overexpression of IL-16 (22) or undefined factors distinct from chemokines (23). Thus, immune, structural and inducible cellular correlates of resistance to infection by HIV were identified. However, many cases of resistance to infection are not accounted for by these factors, and additional means of evading infection despite repeated exposure remain to be discovered.

TABLE 1
Epidemiological similarity between seronegative, high risk study subjects and the larger HIV-resistant sex worker cohort

	Resistant sex worker cohort (n=33)	Study subjects (n=13)	P
Age at cohort entry (years)	31.8±5.0	33.7±5.8	0.38
Sex partners/day (mean)	4.2±1.9	6.6±2.3	0.004
Duration of prostitution at cohort entry (years)	4.1±4.5	6.6±10.8	0.39
Duration of follow-up (years)	4.9±1.7	5.5±1.4	0.36
Episodes of gonorrhea (mean)	0.26±0.20	0.19±0.2	0.40
Episodes of chlamydia infection (mean)	0.04±0.06	0.03±0.05	0.89
Episodes of genital ulcers (mean)	0.06±0.10	0.08±0.07	0.65
Condom use (mean semiquantitative score)	2.5±0.8	3.5±0.4	0.003

Unless otherwise noted, values expressed as ± SD

In this study, we examined CD4⁺ lymphocyte susceptibility to infection in a group of highly HIV-exposed sex workers from Nairobi, Kenya. The subjects had a significantly increased risk of seroconverting but remained uninfected (8). Resistance to infection in the cohort could neither be accounted for by in vitro resistance nor by overproduction of chemokines. Factors produced by CD8⁺ cells from some individuals effectively reduced HIV replication.

MATERIALS AND METHODS

Study subjects: Thirteen cellular samples from the Nairobi sex worker cohort, who were persistently seronegative and remained seronegative after three or more years of follow-up, were available for study. Risk factors for HIV-1 seroconversion in this cohort have been studied since 1985 (8). The risk of infection in the cohort has increased steadily over time; however, the individuals studied remained uninfected as evaluated by serology, immunoblot and polymerase chain reaction (PCR) amplification of *env*, *nef* and *vif* sequences, as described in detail previously (8). The subjects were comparable with the larger resistant sex worker cohort from Nairobi in terms of age, sexual practices and concurrent sexually transmitted diseases (Table 1). Samples from infected members of the larger cohort were available for virus isolation, and control samples were obtained from local and North American unexposed individuals.

Virus isolation, cell preparations and infections: Virus stocks were produced as previously described (23). In brief, CD8⁺ cell-depleted peripheral blood mononuclear cells (PBMCs) from patients were cultured with donor HIV-negative CD4⁺ phytohemagglutinin blasts, and the supernatant was tested biweekly for p24 concentration (Organon Teknika, USA). Two Kenyan nonsyncytium-inducing (NSI) HIV strains and one syncytium-inducing (SI) HIV strain were isolated. CD4⁺ and CD4⁻ cell fractions, or CD8⁺ fractions were immunomagnetically isolated (MiniMACS, Miltenyi Biotec, USA) and exposed to a standardized infection with 10 ng of p24 (corresponding to approximately 500 median tissue culture infective dose [24]) from the respective primary isolate per 10⁶ CD4⁺ phytohemagglutinin blasts. Additionally, CD4⁺ cells from four subjects and six controls were each infected with 1 ng and 0.1 ng of p24. Concentrations of p24 and chemokines (R&D Systems, Minneapolis, Minnesota) in supernatants of uninfected and infected cultures were determined in triplicate biweekly. After three weeks, the remaining cellular fractions were collected and the genomic DNA was extracted.

CD8⁺ cell inhibition studies: To assess cellular suppression of viral replication, CD8⁺ cells were added to autologous CD4⁺ cells in a 1 to 4 ratio immediately after infection. The supernatants were replenished biweekly, and p24 levels in uninfected cell cultures, infected CD4⁺ cell cultures and CD4⁺-CD8⁺ cocultures were determined biweekly.

CCR5 genotyping: High molecular weight DNA was subjected to PCR amplification with primers spanning the described 32 base pair deletion in the gene coding for CCR5 (16). The amplicons were separated in 3% agarose gels.

Statistical analysis: Differences between the control infections and the sample population were evaluated with the Wilcoxon

TABLE 2

Viral p24 concentrations in CD4⁺ culture supernatants from persistently seronegative and control individuals in vitro infected with primary HIV isolates

	Inoculum (ng)	p24 concentration (pg/mL) – Day 7				p24 concentration (pg/mL) – Day 14			
		$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
NSI-1		Subjects (n=9)		Controls (n=6)		Subjects (n=9)		Controls (n=6)	
	10	1151	242	1829	198	2315	309	2431	222
NSI-2		Subjects (n=4)		Controls (n=6)		Subjects (n=4)		Controls (n=6)	
	10	15,875	2750	19,354	3335	20,093	3124	24,654	3917
	1	7441	884	8098	1231	17,049	2323	17,814	2534
	0.1	2398	450	2412	690	10,005	1438	9465	1982
SI		Subjects (n=13)		Controls (n=6)		Subjects (n=13)		Controls (n=6)	
	10	21,627	8224	23,252	8482	30,982	5908	33,293	6189

NSI Nonsyncytium-inducing HIV; SI Syncytium-inducing HIV

signed-rank test for nonparametric samples (25). Inhibitions of viral replication were compared using the paired *t*-test (25).

RESULTS

Susceptibility of CD4⁺ cells from resistant subjects to in vitro infection: Two NSI isolates and one strongly SI virus were isolated. Purified CD4⁺ cells from 13 members of the resistant cohort, and one local and five Caucasian control individuals were infected with the primary isolates. Although the individual viruses differed in their replicative ability, no significant differences were observed between the sample population and controls with either of the NSI viruses, dilutions of an NSI virus or with the SI virus (Table 2). Similar results were obtained in four subjects with clade B North American primary viral isolates (data not shown).

Beta-chemokine production by CD4⁺ cells: To determine whether beta-chemokine production is associated with reduced or enhanced infectivity of CD4⁺ cells, the concentrations of MIP-1-alpha, MIP-1-beta and RANTES were determined. No significant differences between infected and uninfected cultures were noted. Rather, chemokine production was characteristic for each individual, regardless of whether the cells were infected (Figure 1).

CD8⁺ cell-mediated viral suppression: To assess the effect of CD8⁺ cells on virus replication, autologous, unstimulated CD8⁺ cells were added to CD4⁺ cells immediately following infection. In four individuals, profound inhibition ($\bar{x}$ =93.4%) of NSI viruses was observed. There was little reduction ($\bar{x}$ =2.7%) of SI virus replication. CD8⁺ cells from unexposed individuals suppressed replication of NSI viruses ($\bar{x}$ =90.1%) and SI viruses ($\bar{x}$ =62.3%). Thus, in some members of this cohort, CD8⁺ cells specifically suppressed NSI viral isolates.

CCR5 genotyping: No deletions in the amplified segment of CCR5 were identified in the seronegative or seropositive subjects (data not shown).

DISCUSSION

In this study, the susceptibility of CD4⁺ cells from a group of persistently HIV-seronegative, commercial sex workers from Nairobi, Kenya, was investigated (8). The odds ratio for sero-

Figure 1) Beta-chemokine production by CD4⁺ lymphocytes varies between individuals, independent of infection. CD4⁺ cells were uninfected (UI), infected with syncytium-inducing (SI) or nonsyncytium-inducing (NSI) viruses, and the concentration of macrophage inflammatory protein (MIP)-1-alpha (MIP-1α), MIP-1-beta (MIP-1β) and RANTES in supernatants was determined by immunoassay

conversion for all participants in the study increased with the duration of prostitution, and the probability of remaining seronegative decreased exponentially over time (8). However, in the subgroup reported on by Fowke et al (8), a protective effect that increased with the duration of prostitution was observed. This is a very unusual situation, and because these individuals present a unique opportunity to study apparent natural resistance to HIV infection, we sought to evaluate in vitro correlates of infectivity or resistance.

Samples from 13 randomly selected individuals were available for study; they did not vary from other 'resistant' members of the cohort significantly with respect to age, duration of prostitution or sexually transmitted diseases (Table 1). The subset had a greater number of sex partners per day and concomitantly higher condom use. Neither of these factors was expected to influence cellular characteristics pertinent to this study. In vitro studies revealed no constitutive barriers to HIV

infection or replication. These findings exclude the presence of a structural defect such as an altered receptor or coreceptor for the virus, and suggest that CD4⁺ cells from the subjects were inherently susceptible to infection and competent to allow for viral replication. No difference regarding the syncytial phenotype was observed, indicating that the change in cell tropism that is commonly associated with a switch from NSI to SI phenotype may proceed unimpaired in the cohort.

Production of chemokines by CD4⁺ cells was not associated with resistance to infection by NSI and SI viruses in this study. MIP-1 and MIP-1 production exceeded 40,000 pg/mL in only one subject, and this was independent of infection. There was much individual variation in chemokine production, similar to what has been described for CCR5 expression (26). In other studies, excessive production of the chemokines MIP-1- α , MIP-1- β and RANTES (30,000 to 100,000 pg/mL) correlated with a reduction or absence of CD4⁺ cell infection, and addition of 200,000 pg/mL of all three chemokines was required to block viral replication in CD4⁺ cells (20,27). Thus, competition for binding to CCR5 appears to require very high amounts of all three chemokines in combination, and autologous production at levels exceeding those observed in this study may be necessary for a protective effect. These findings do not preclude protection specifically mediated at mucosal sites by nonlymphoid production of chemokines or other factors. Interestingly, synthesis of the above chemokines has been associated with a Th1 immune response induced by a bacterial antigen (28). Th1 immune responses correlated with relative protection from HIV infection and from disease progression, while in the later stages of HIV disease, cellular defects became more pronounced and antibody production persisted, characteristic of Th2 immune responses (12,29). The subjects in the present study were exposed to numerous other sexually transmitted organisms (30). Therefore, it is conceivable that induction of a Th1 immune response and local secretion of chemokines could impair the establishment of HIV infection at the mucosal site.

Concurring with previous reports that the CCR5 $\Delta 32$ phenotype is unique to individuals of Caucasian descent (31), nei-

ther resistance to infectability with viruses binding to CCR5 nor the described 32 base pair deletion in CCR5 were identified in subjects in the present study. Kenyan viral isolates belong predominantly to clade A and a smaller proportion to clade D (32), but HIV-1 subtypes A, B, C, D and E and group O viruses all rely on CCR5 for viral entry and do not replicate in cells from CCR5 $\Delta 32$ homozygous individuals (33). Thus, resistance in this cohort was not due to properties of CD4⁺ cells.

Noncytolytic CD8⁺ cell suppression of viral replication has been described in long term nonprogressing patients (34), and correlated with CD4 count and a lack of disease progression (35,36). Although high levels of the beta-chemokines inhibit infection by NSI isolates (14), discussion continues regarding the complete identity of the CD8⁺ cell-derived factors (37). The suppressive effect observed by coculture with CD8⁺ cells in this study was specific to NSI isolates and, therefore, may have been partially mediated by CD8⁺ cell production of chemokines. However, only one CD8 cell per four CD4 cells was present, and the CD8⁺ cells were not previously stimulated in vitro, suggesting that very high constitutive production of chemokines is required. Thus, it is likely that factors other than those described in this study contribute to in vitro and in vivo resistance.

The contribution of cytolytic immune responses to HIV resistance in this cohort of persistently seronegative individuals was not assessed; instead, CD8⁺ cell nonlytic effector functions were evaluated. Previous studies have identified cytolytic T cell reactivity in a small percentage of exposed sex workers from Gambia (9), and in members of the cohort reported on here (10). Thus, resistance to infection may be due to several factors, including cytolytic and suppressive functions of CD8⁺ cells. Because there was no inherent interference with infection of isolated CD4⁺ cells, there are likely to be factors modulating or preventing infection at the mucosal site of initial exposure. CD8⁺ cells may exert their most profound effect by limiting an infection to a degree sufficient for generating major histocompatibility complex class I-restricted cytotoxic T lymphocytes, which are then able to eliminate virally infected cells at a localized site.

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