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No Difference in *in Vitro* Susceptibility to HIV Type 1 between High-Risk HIV-Negative Ethiopian Commercial Sex Workers and Low-Risk Control Subjects

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ABSTRACT

Host factors such as increased β-chemokine production, HIV-1 coreceptor expression level, and HIV-1 coreceptor polymorphism have been thought to influence susceptibility to HIV-1 infection. To determine the protective role of these factors in Ethiopians who remained HIV-1 uninfected, despite multiple high-risk sexual exposures, we studied 21 Ethiopian women who had been employed as commercial sex workers (CSWs) for five or more years. The HIV-1-resistant CSWs were compared with low-risk age-matched female controls who had a comparable $CD4^+$ cell percentage and mean fluorescence intensity (MFI). Genetic polymorphism in the CCR5, CCR2b, or SDF-1 genes appeared not to be associated with resistance in the Ethiopian CSWs. Expression levels of CCR5 and CXCR4 on naive, memory, and total $CD4^+$ T cells tended to be higher in the resistant CSWs, while the production of β-chemokines RANTES, MIP-1α, and MIP-1β by phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) was lower compared with low-risk HIV-1 negative controls. *In vitro* susceptibility of PHA-stimulated PBMCs to primary, CCR5-restricted, Ethiopian HIV-1 isolates was comparable between resistant CSWs and low-risk controls. *In vitro* susceptibility was positively correlated to $CD4^+$ cell mean fluorescence intensity and negatively correlated to CCR5 expression levels, suggesting that infection of PBMCs was primarily dependent on expression levels of CD4 and that CCR5 expression, above a certain threshold, did not further increase susceptibility. Our results show that coreceptor polymorphism, coreceptor expression levels, β-chemokine production, and cellular resistance to *in vitro* HIV-1 infection are not associated with protection in high-risk HIV-1-negative Ethiopian CSWs.

INTRODUCTION

THE IDENTIFICATION OF INDIVIDUALS who remain HIV-1 uninfected despite high-risk exposures has suggested that host factors might contribute to resistance for HIV-1 infection.[1–12]

First, although it is not absolute,[13,14] a homozygous 32-bp deletion (Δ32) in the gene encoding CCR5, the coreceptor for macrophage-tropic (M-tropic) HIV-1 isolates,[15–17] has been found to be associated with HIV-1 resistance in Caucasian individuals.[5,18–20] Furthermore, individuals with a heterozygous genotype are reported to exhibit slow disease progression.[21,22] The allelic frequency of this deletion is low in Africa.[23] Second, reduced *in vitro* HIV-1 susceptibility of $CD4^+$ T cells of exposed but uninfected individuals was found to be associated with low surface expression of CCR5 on $CD4^+$ T cells and high production of the β-chemokines RANTES, MIP-1α, and MIP-1β.[4,6] In addition to these host factors, there are others, such as certain HLA alleles,[24,25] the presence of HIV-1-specific cytotoxic T cell responses,[8,26–28] and HIV-1-specific mucosal IgA,[7,9] that have been suggested as protective mechanisms against HIV-1 infection.

In Ethiopia the HIV-1 epidemic started relatively late, the

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first HIV-1-positive sera being detected in 1984.[29] Since then the epidemic has been spreading fast and by 1998 it had reached a prevalence ranging between 7 and 20% in the 15 to 49-year age group in urban populations.[30,31] Surveys conducted between 1994 and 1998 in Addis Ababa, the capital of Ethiopia, showed that the HIV-1 prevalence among commercial sex workers (CSWs) was 45% in 1994 and has reached up to 74% in 1998.[31] This increase in prevalence is an indication that there is a continuous exposure to HIV-1 among CSWs and that the epidemic is gradually spreading in this risk group. Therefore, individuals who work as CSWs for a long time period are potentially at high risk for acquisition of an HIV-1 infection. Hence, sex workers who were still HIV-1 uninfected in 1998 despite many years of sex work, may represent one of the most appropriate groups to study to determine whether there are biological factors conferring resistance to HIV-1 infection in the Ethiopian population.

In this study we investigated individuals who remained HIV-1 negative despite their work as CSWs for five or more years. The *in vitro* susceptibility of their peripheral blood mononuclear cells (PBMCs) to primary HIV-1 isolates from HIV-1-positive CSWs residing in the same area was assessed and gene polymorphisms and expression levels of HIV-1 coreceptors as well as *in vitro* β-chemokine production were determined.

MATERIALS AND METHODS

Subjects

The high-risk HIV-1-negative group consisted of 21 female CSWs living in Addis Ababa who reported to have been working as CSWs for five or more years. The mean number of years of employment as sex worker was 13 years. Most were already in sex work when the epidemic was starting (1984–1988) and when condoms were not yet widespread. None was using condoms consistently (100%) with clients at the time of the survey. Eighteen of 21 (86%) took drugs for treatment of vaginal discharge in the past year and 3 of 21 (14%) took drugs for the treatment of genital ulcer. For controls, six age-matched, low-risk HIV-1-negative females participating in the Ethio-Netherlands AIDS Research Program (ENARP) Akaki cohort studies were enrolled. The low-risk women reported themselves as either engaged in a monogamous relation or as never having had sexual contacts. There was no history of sexually transmitted diseases (STDs) with these women. All study subjects gave their informed consent to participate in the study. Ethical clearance for the study was obtained from both the Ethiopian Health and Nutrition Research Institute (EHNRI, Addis Ababa, Ethiopia) and National Ethical Clearance Committees.

HIV-1 testing

HIV-1 testing was performed by using a combination of a rapid assay (HIV-SPOT; Genelabs Diagnostics, Singapore) and an enzyme-linked immunosorbent assay (ELISA) (Vironostika HIV Uni-form plus O; Organon Teknika, Boxtel, The Netherlands). As indicated by the manufacturers, the specificity of the serologic tests, used for screening, for HIV-1 groups M and O is 99.9%.

HIV viral load in plasma was determined by using a nucleic acid sequence-based amplification (NASBA) kit (Nuclisens, Organon Teknika). All selected high-risk CSWs had no detectable viral load in plasma.

Syphilis serology

Syphilis serology was performed by *Treponema pallidum* particle agglutination assay (TPPA) (Serodia-TPPA; Fujirebio, Tokyo, Japan) and rapid plasma reagin assay (RPR-nosticon II; Organon Teknika), according to the manufacturer instructions.

Peripheral blood mononuclear cells isolation

PBMCs were isolated from venous blood collected in EDTA Vacutainers by density gradient centrifugation on Ficoll-Hypaque and were viable frozen in liquid nitrogen until shipment on dry ice to the CLB (Centraal Laboratorium van de Bloedtransfusiendienst, Amsterdam, The Netherlands) for further analysis.

Virus isolation and characterization

Virus clones were isolated from PBMCs of six HIV-1-positive CSWs by cocultivation with healthy donor PBMCs under limiting diluting conditions, as described previously.[32] Briefly, donor PBMCs, which were prestimulated for 3 days with phytohemagglutinin (PHA) were cocultivated with increased numbers of PBMCs from HIV-1^+ CSWs in 96-well microtitration plates (range, 5000–20,000 cells per coculture). The presence of HIV-1 p24 antigen in each microculture supernatant was tested every week, using an in-house p24 antigen capture enzyme-linked immunosorbent assay.[33] At the same time one-third of the culture was transferred to new 96-well plates and mixed with fresh donor PBMCs, which were prestimulated as described above for further propagation until biological virus clones were obtained. Virus stocks were grown on PHA-stimulated PBMCs from the p24-positive wells and stored at -70°C until use. The MT2 assay was used to determine the syncytium-inducing phenotype of the viral isolates.[34] Coreceptor usage of the biological virus clones was determined on human astroglioma U87 cell lines expressing CD4 and either CCR5, CXCR4, or CCR3, as described previously.[35] Briefly, 2×10^4 cells of the various U87 cell lines were seeded in 96-well plates and inoculation with 10^3 50% tissue culture infectious doses ($TCID_{50}$) of each biological clone was done after 24 hr. The inoculated cells were incubated for another 24 hr and then washed with phosphate-buffered saline (PBS) and 200 μl of fresh medium was added. On day 7 the cells were detached by trypsinization and transferred to 24-well plates. p24 production was measured in culture supernatant sampled on days 7, 14, and 21, respectively. The viral clones were also used to infect PBMCs from individuals with wild-type and homozygous CCR5Δ32 genotype, to analyze coreceptor usage on primary cells.

Determination of sensitivity of the viral isolates to β-chemokines: RANTES, MIP-1α, and MIP-1β

Donor PBMCs, prestimulated for 3 days with PHA, were incubated with serial dilutions of RANTES, MIP-1α, and MIP-1β, ranging from 15.625 to 250 ng/ml, in 96-well plates for 3

TABLE 1. CHARACTERISTICS OF STUDY SUBJECTS

	CSWs (n = 21)	*Controls (n = 6)*
Age (years)	33 (25–45)[a]	32 (25–35)
Period of CSW employment (years)	14 (5–25)	0
Syphilis positive	52%	0%
$CD4^+$ T cell %	39 (24–50)	40 (11–47)
$CD4^+$ T cell MFI	107 (74–160)	111 (101–127)

Abbreviation: MFI, mean fluorescence intensity.
[a]Values represent median and 95% range.

hr at 37°C. Cells were then inoculated with 5- and 25-fold dilutions of 10^3 $TCID_{50}$ of 4 nonsyncytium-inducing (NSI) biological clones from HIV-1^+ CSWs, 1 NSI isolate from an Ethiopian AIDS patient (PHD50), and 1 NSI isolate from a Dutch subject (Dutch NSI). p24 production was monitored over 14 days. PHA-stimulated PBMCs that had not been incubated with chemokines but that were inoculated with the different HIV-1 variants provided reference values for maximum virus production.

Measurement of β-chemokine production by PHA-stimulated PBMCs

Cryopreserved PBMCs from CSWs and controls were cultured in 24-well plates at a concentration of 1×10^6 cells/ml in the presence of PHA for 3 days. Subsequently, culture supernatant from each culture was sampled and stored at −20°C until analysis. The production of RANTES, MIP-1α, and MIP-1β in the culture supernatant was determined by using commercial ELISA kits, according to the instructions of the manufacturer (R&D Systems. Abingdon, UK).

Measurement of CCR5 and CXCR4 expression

Quantification of $CD4^+$ T cells and their naïve ($CD45RA^+$) and memory ($CD45RO^+$) subsets expressing CCR5 and CXCR4 was performed by three-color flow cytometry, either on whole blood or on isolated fresh PBMCs, using combinations of peridinin chlorophyll protein (PerCP)-conjugated CD4 monoclonal antibodies (MAbs) with fluorescein isothiocyanate (FITC)-conjugated CD45RA or CD45RO MAb and phycoerythrin (PE)-conjugated CCR5 (2D7) or CXCR4 (12G5) MAb. MAbs used to distinguish CCR5 and CXCR4 were purchased from PharMingen (La Jolla, CA). All other MAbs were purchased from Becton Dickinson (San Jose, CA). The stained samples were analyzed the same day, using a FACScan flow cytometer with CellQuest software. A live gate was set around the $CD4^+$ T cells in order to acquire a minimum of 1500 $CD4^+$ T cells for analysis.

Coreceptor polymorphism analysis

Genomic DNA was isolated from cryopreserved PBMCs (Qiagen blood kit; Qiagen, Westburg, Germany). CCR5 Δ32-bp hetero- or homozygosity was analyzed by polymerase chain reaction (PCR), using primers flanking the Δ32 deletion in the CCR5 gene.[36] CCR2b 641 and SDF-1 3′A genotyping was performed as described previously, using *Bsa*BI and *Msp*I restriction fragment length polymorphism (RFLP)-PCR analysis, respectively.[37,38]

In vitro *susceptibility testing of PBMCs*

Cryopreserved PBMCs from CSWs and controls were thawed and stimulated with PHA for 3 days. Susceptibility of CSW and control PBMCs to four viruses isolated from HIV-1-infected CSWs was determined by using several 5-fold dilutions of the viruses, starting with 1000 $TCID_{50}$, and measurement of p24 production over a 2-week time period. The difference in virus titer expressed as 50% tissue culture infectious dose per milliliter of supernatant was used as a measure of *in vitro* susceptibility.

Statistical analysis

Statistical analyses were performed with the STATA program (Stata, College Station, TX). Biological factors and susceptibility were compared between two groups, using nonparametric methods (Mann–Whitney *U* test). Spearman correlation coefficients (*r*) were used to describe correlations between continuous variables. Mutation frequencies for HIV-1 coreceptors were compared between two groups by Fisher exact test.

TABLE 2. CORECEPTOR USE OF THE HIV-1 BIOLOGICAL CLONES ISOLATED FROM HIV-1^+ CSWS

	PBMCs		*U87-CD4 coexpressing*		
Isolate	*CCR5 WT*	*CCR5 Δ32/Δ32*	*CCR3*	*CXCR4*	*CCR5*
50.2A3	+	−	−	−	+
44.1E7	+	−	−	−	+
6.2D4	+	−	−	−	+
6.1B1	+	−	−	−	+

Abbreviation: WT, wild type.

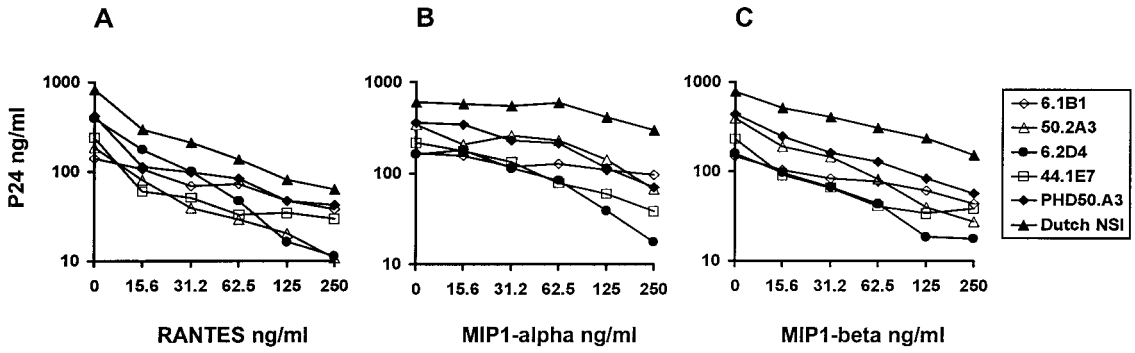

FIG. 1. Dose-dependent inhibition of *in vitro* replication of five Ethiopian (four from HIV-1^+ CSWs, 6.1B1, 6.2D4, 50.2A3, and 44.1E7; one from AIDS patient, PHD50.A3) and one Dutch (Dutch NSI) HIV-1 biological clones by (**A**) RANTES, (**B**) MIP-1α, and (**C**) MIP-1β. PBMCs were infected in the presence of β-chemokines at a concentration ranging from 15.625 to 250 ng/ml as described in Materials and Methods. p24 production, as a measure of HIV-1 replication, was determined 7 days postinfection.

RESULTS

A total of 372 CSWs reporting at two health centers in Addis Ababa in 1998 were tested for HIV-1 antibodies and 275 (74%) appeared to be HIV-1 positive. The blood donation was linked to a questionnaire but not to the study subjects. Of the 97 HIV-1-negative CSWs, 21 subjects were selected who reportedly worked as CSWs for 5–25 years. Table 1 shows some characteristics of the study subjects, in comparison with six age-matched women of low-risk behavior, as controls. As can be seen, the $CD4^+$ T cell percentages and CD4 expression levels were comparable between the two groups. More than half of the HIV-1-negative CSWs showed positive syphilis serology, in contrast to 0% of the control group.

Characterization of HIV-1 isolates from CSWs

First, four primary HIV-1 isolates were derived from three different HIV-1-positive CSWs from the same study area as the HIV-1-negative CSWs. Coreceptor usage of these primary HIV-1 isolates was determined on U87 cell lines and on PBMCs from individuals with CCR5 wild-type and CCR5 $\Delta 32/\Delta 32$ genotype. As shown in Table 2, the four isolates used exclusively CCR5 as a coreceptor. Sequence analysis of the isolates revealed that they all belong to subtype C virus (data not shown).

β-Chemokine sensitivity of HIV-1 isolates

β-Chemokine sensitivity of the isolates was tested in the presence of each of the β-chemokines RANTES, MIP-1α, and MIP-1β at concentrations ranging from 15.625 to 250 ng/ml in PHA-prestimulated PBMCs. As measured by p24 production, all four isolates were sensitive to all three β-chemokines and there was no significant variation in the sensitivity of the different isolates to any of the β-chemokines (Fig. 1A–C). Fifty percent inhibition was evident at a concentration of 32.25 ng/ml for RANTES and MIP-1α against all the Ethiopian isolates. For MIP-1β, the 50% inhibition concentration was two times higher (62.5 ng/ml). However, the Dutch NSI control virus isolate appeared less sensitive in comparison, especially to MIP-1β (Fig. 1A–C). When cultured in the absence of β-chemokines, all the isolates showed the same growth kinetics over 14 days (Fig. 2A–C). In addition, although lower level p24 production was observed in the presence of RANTES at 125 ng/ml, MIP-1α and MIP-1β growth kinetics were still comparable (Fig. 2A–C).

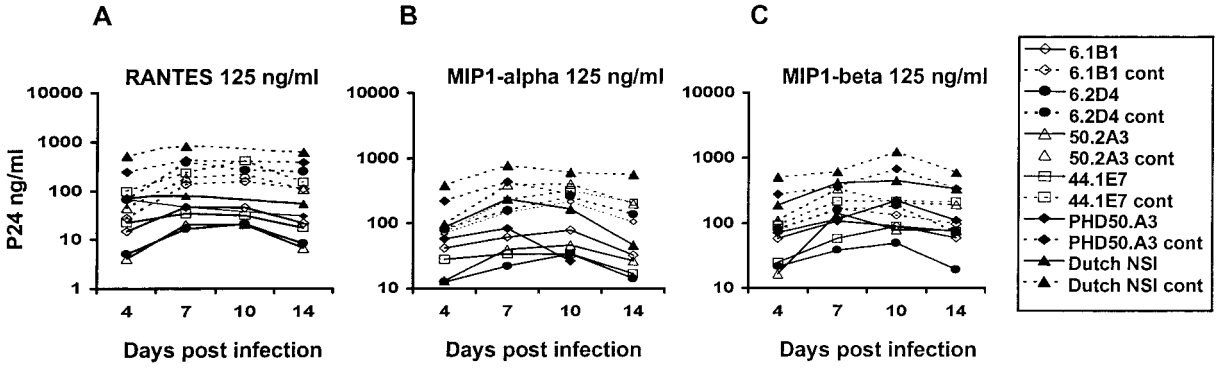

FIG. 2. Growth kinetics of the four HIV-1 biological clones isolated from HIV-1^+ CSWs and two other biological clones (one Ethiopian and one Dutch NSI) in the presence or absence of β-chemokines over a 14-day period. PBMCs were infected in the absence (dotted lines) or presence (solid lines) of a 125-ng/ml concentration of (**A**) RANTES, (**B**) MIP-1α, and (**C**) MIP-1β.

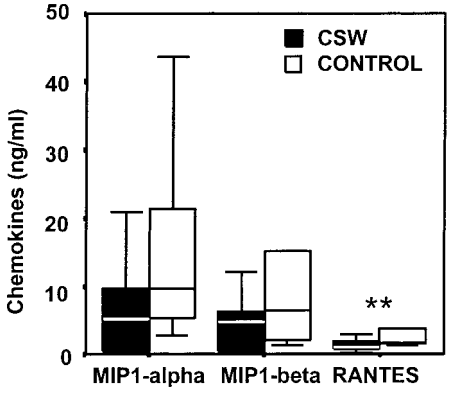

FIG. 3. Box plots of RANTES, MIP-1α, and MIP-1β production by 3-days PHA-stimulated PBMCs from CSWs (solid boxes) and controls (open boxes). The boxes correspond to 50th percentiles and horizontal bars within these boxes indicate the median. The 95% percentile values are indicated by the bars. $^{**}p < 0.05$ by Mann–Whitney *U* test.

Coreceptor mutation analysis

Samples from all study participants were analyzed for the presence of the 32-bp deletion in CCR5, the CCR2b 64I genotype, and the 3′ A SDF-1 mutation. All high-risk HIV-1-negative CSWs tested ($n = 16$) had a CCR5 wild-type genotype. However, mutations were detected for the other coreceptor genes: 37.5% heterozygosity and 12.5% homozygosity for CCR2b 64I, 43.7% heterozygosity and 12.5% homozygosity for 3′A SDF-1. Since the control group was small, the frequencies of the mutations observed were compared with the frequencies found in HIV-1-negative participants of the ENARP Akaki and Wonji cohort sites (CCR2b [$n = 106$] heterozygous: 31%, homozygous: 7%; 3′A SDF-1 [$n = 111$] heterozygous: 29%, homozygous: 6%[38a]). The distribution of the mutation frequencies was not significantly different between the two groups.

β-Chemokine production

RANTES, MIP-1α, and MIP-1β production was measured in PBMCs from CSWs and controls after 3 days of stimulation with PHA. Only for RANTES was a significantly higher production measured in the control women versus the HIV-1-negative CSWs. A tendency could be seen for higher production of MIP-1α and MIP-1β in the control women compared with the CSWs (Fig. 3).

Surface expression levels of CCR5 and CXCR4

Three-color FACS analysis was performed on fresh, unstimulated PBMCs from CSWs and controls to determine CCR5 and CXCR4 expression levels on naive and memory $CD4^+$ T cells. Table 3 shows that there is a tendency for higher expression of CCR5 and CXCR4 in all the subsets in the CSWs compared with the controls. However, the mean fluorescence intensity (MFI) only of CXCR4-expressing naive ($CD45RA^+$) and total $CD4^+$ T cells was significantly higher in the CSWs than in the controls.

In vitro *susceptibility of PMBCs from high-risk CSWs compared with controls*

The four HIV-1 primary isolates were used to infect PBMCs from CSWs and controls to test for *in vitro* susceptibility. Figure 4A shows the log($TCID_{50}$/ml) in the PBMCs from CSWs and controls 14 days after inoculation with the four HIV-1 biological clones. For none of the four viruses tested was a significant difference in titer detected between CSWs and controls, thus indicating a comparable susceptibility to *in vitro* HIV-1 infection. The amount of p24 produced after 14 days of infection is shown in Fig. 4B. Although there was no significant difference in p24 production level between the two groups, a higher proportion of control women showed p24 levels above an arbitrary threshold of 150 ng/ml (data not shown).

Correlation of β-chemokine production with susceptibility

Although RANTES production after 3 days of PHA stimulation was significantly higher in the control group than in the HIV-1-negative CSWs, lower *in vitro* susceptibility, as measured by log($TCID_{50}$/ml), was not observed in this group.

We have calculated Spearman correlation coefficients of production of individual β-chemokines RANTES, MIP-1α, and MIP-1β with susceptibility, as measured by mean log($TCID_{50}$/ml), obtained for each of the four HIV-1 biologi-

TABLE 3. CORECEPTOR EXPRESSION LEVELS ON NAIVE AND MEMORY $CD4^+$ T CELLS OF HIV-1-NEGATIVE CSWS AND CONTROLS

Coreceptor/T cell subset	*CSWs* (n = *21*)		*Controls* (n = *6*)	
	%	*MFI*[a]	*%*	*MFI*
CCR5				
Naive ($CD45RA^+$)	16 (1–38)	48 (30–220)	14 (8–26)	42 (29–59)
Memory ($CD45R0^+$)	33 (21–87)	51 (28–102)	27 (23–39)	54 (44–57)
Total $CD4^+$	33 (16–57)	52 (30–105)	27 (20–39)	52 (38–59)
CXCR4				
Naive ($CD45RA^+$)	82 (66–95)	177 (69–363)[b]	79 (64–85)	104 (79–148)
Memory ($CD45R0^+$)	71 (56–98)	205 (77–506)[b]	68 (58–80)	102 (58–131)
Total $CD4^+$	73 (62–87)	199 (71–469)[b]	69 (58–84)	104 (64–133)

[a]MFI of positive cells is shown.
[b]significantly higher compared with controls, $p < 0.05$ by Mann–Whitney U test.

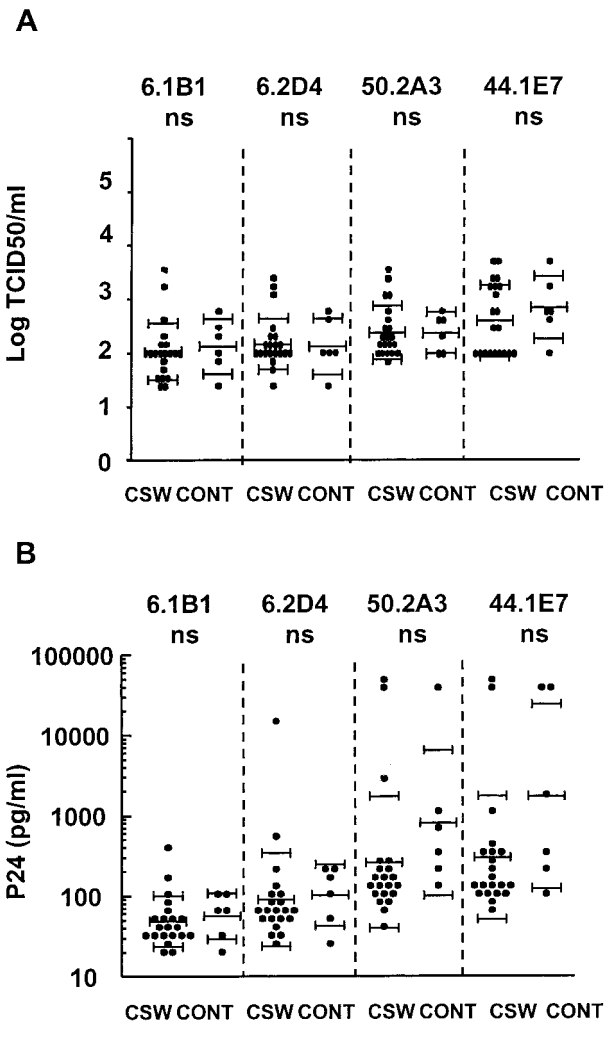

FIG. 4. Susceptibilities of PBMCs from CSWs and control (cont) subjects to infection by the four HIV-1 biological clones expressed as (**A**) log($TCID_{50}$/ml) and (**B**) p24 production. Each dot represents PBMCs from a single subject. Bars indicate the means and the standard deviations.

cal clones. As shown in Fig. 5A, RANTES production levels appeared to be positively correlated ($r = 0.45$, $p < 0.05$) with mean log($TCID_{50}$/ml). The production levels of MIP-1α and MIP-1β were not correlated significantly ($r = 0.24$, p = NS and $r = 0.08$, p = NS, respectively) with mean log($TCID_{50}$/ml) (Fig. 5B and C).

Correlation of CCR5 and CD4 expression levels with susceptibility

Since the difference in the percentage of cells expressing CCR5 was not associated with a difference in susceptibility between the two groups of CSWs and controls, it was tested whether there was a correlation of CCR5 expression levels on $CD4^+$ T cells, or of the MFI level of CD4 expression, with HIV-1 susceptibility. CCR5 expression level was found to be negatively correlated ($r = -0.48$, $p < 0.05$) with mean log($TCID_{50}$/ml), calculated as the average log($TCID_{50}$/ml) of the four HIV-1 biological clones tested. The correlation remained significant ($r = -0.40$, $p < 0.05$) even when outliers were excluded from the analysis (Fig. 5D). In contrast, the level of expression of CD4 showed a significant positive correlation ($r = 0.59$, $p = 0.001$) with mean log($TCID_{50}$/ml) (Fig. 5E).

DISCUSSION

The existence of biological factors that influence susceptibility to HIV-1 infection was studied in 21 HIV-1-negative high-risk Ethiopian CSWs. These women were selected from a population of 372 CSWs attending an STD clinic in Addis Ababa, on the basis of the absence of HIV-1-specific antibodies and a minimum of 5 years of employment as a CSW. The overall HIV-1 antibody prevalence in the CSW population was 74%. In addition, 52% of the women in the HIV-1-negative CSW group were found to be positive for antibodies to syphilis, indicating high-risk sexual behavior. Therefore, the presence of biological factors conferring resistance to the HIV-1-negative high-risk CSWs could be anticipated.

A comparison of the high-risk HIV-1-negative CSWs with Ethiopian females at low risk for HIV-1 infection revealed no significant differences in *in vitro* HIV-1 susceptibility, β-chemokine production, and HIV-1 coreceptor expression levels. This is in agreement with a report on Kenyan CSWs, where no association was found between HIV-1 resistance and decreased cellular susceptibility or enhanced β-chemokine production.[39] Our data extend these findings in some important aspects. In contrast to the Kenyan study, where laboratory-adapted strains of HIV-1 were used to test *in vitro* susceptibility of PBMCs, we used primary isolates from HIV-1-positive CSWs residing and working in the same area as the HIV-1-negative CSWs.

All primary HIV-1 isolates had a non-syncytium-inducing phenotype, used CCR5 exclusively as a coreceptor, and were sensitive to β-chemokines *in vitro*, indicating that the observed lack of association between susceptibility, CCR5 expression, and β-chemokine production could not be attributed to the phenotype of the viruses.

Increased β-chemokine production, which downregulates HIV-1 coreceptors, is among the host factors reported to be associated with resistance to HIV-1 infection.[40,41] In contrast to what could have been expected, the HIV-1-negative high-risk CSWs of our study showed a significantly lower RANTES production and a tendency to produce less MIP-1α and MIP-1β compared with control women. However, these observed differences in the production of β-chemokine were not associated with a difference in *in vitro* susceptibility between the two groups. Thus, enhanced β-chemokine production levels did not reduce *in vitro* susceptibility in our study population. Instead, an overall positive correlation between RANTES production and susceptibility was found, when all subjects were analyzed together. An explanation for this could be that higher production of RANTES on PHA stimulation is possibly a reflection of the presence of more activated cells in the CSWs. But also, the amount of RANTES produced was much lower than the concentration needed for significant inhibition of HIV-1.

Expression levels of CCR5 and CXCR4 were determined not only on total $CD4^+$ T cells but also on naive ($CD45RA^+$) and

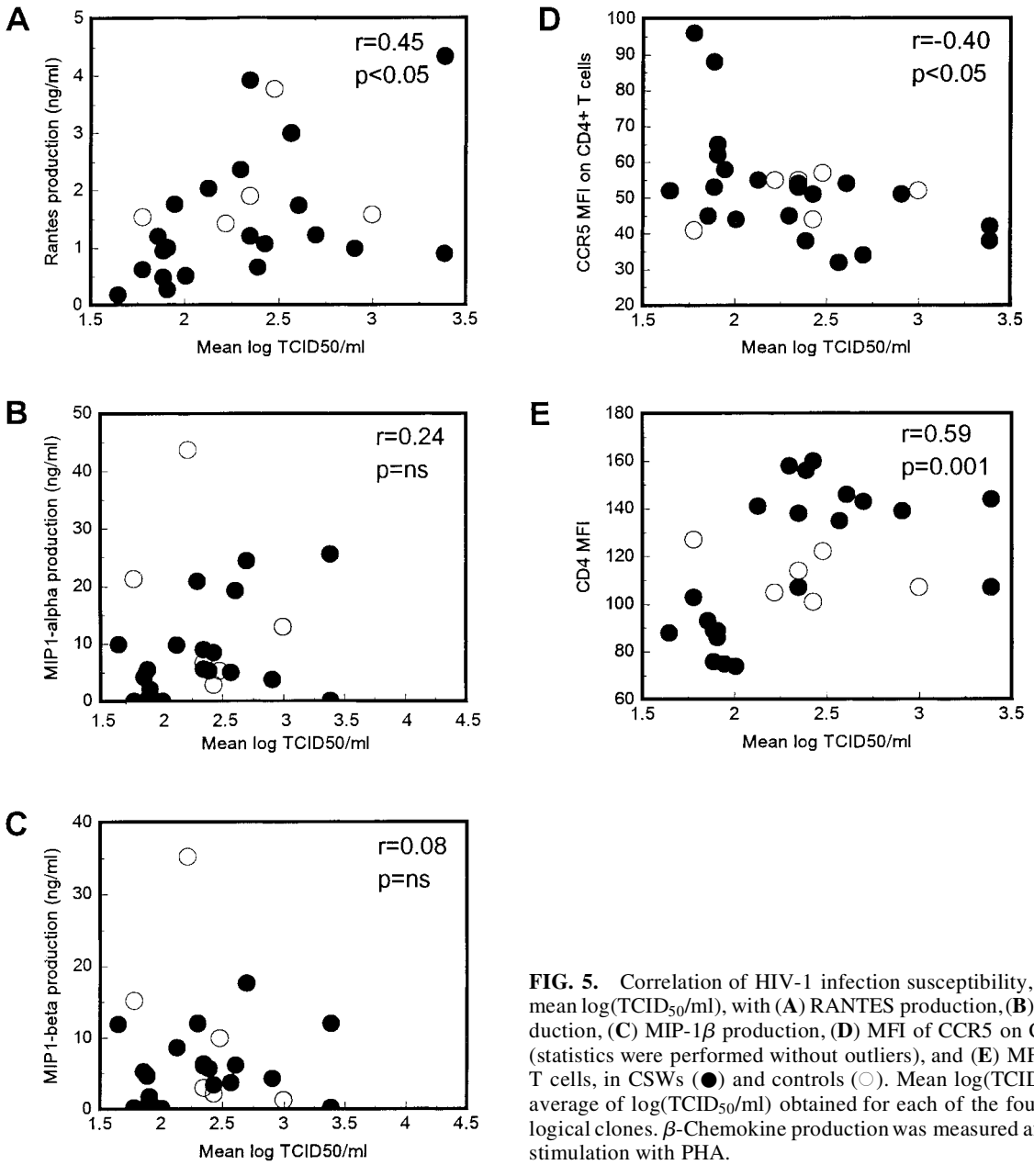

FIG. 5. Correlation of HIV-1 infection susceptibility, expressed as mean log($TCID_{50}$/ml), with (**A**) RANTES production, (**B**) MIP-1α production, (**C**) MIP-1β production, (**D**) MFI of CCR5 on $CD4^+$ T cells (statistics were performed without outliers), and (**E**) MFI of CD4 on T cells, in CSWs (●) and controls (○). Mean log($TCID_{50}$/ml) is the average of log($TCID_{50}$/ml) obtained for each of the four HIV-1 biological clones. β-Chemokine production was measured after 3 days of stimulation with PHA.

memory ($CD45R0^+$) T cell subsets. High-risk HIV-1-negative CSWs tended to have higher CCR5 and CXCR4 expression in all subsets, although the difference was only significant for MFI of CXCR4-positive naive, memory, and total $CD4^+$ T cells. As with found for β-chemokine production, the differences in coreceptor expression were not associated with differences in *in vitro* susceptibility. The higher expression levels of CCR5 and CXCR4 in CSWs compared with control women might be a reflection of increased immune activation due to recurrent infections associated with their high-risk behavior.[42,43] Although CCR5 expression in our study population was not associated with the Δ32-bp mutation in the CCR5 gene, we cannot exclude the possibility that other mutations of the CCR5 gene, i.e., in the promotor region, might contribute to the observed differences.

A study by Pesenti *et al.* on *in vitro* susceptibility of macrophages implicated that a low concentration of CCR5 is sufficient for efficient HIV-1 infection.[44] The expression level of CD4 was suggested to be the primary factor for susceptibility. Hence, CCR5 expression levels above a certain threshold would not influence susceptibility. In concordance with their observation in macrophages, we found a positive correlation between CD4 expression level and *in vitro* susceptibility of PBMCs. This finding might explain the lack of association between β-chemokine production, coreceptor expression, and *in vitro* susceptibility. Surprisingly, in our study a negative correlation between CCR5 expression level on total $CD4^+$ T cells and *in vitro* susceptibility was seen. This is in contrast to previous data indicating that low CCR5 expression levels correlated with reduced *in vitro* susceptibility.[6,43,45] However, high

CCR5 expression levels *in vivo* might not necessarily lead to high expression after *in vitro* stimulation.[43] In fact, cells that have already been activated *in vivo* might be less responsive to further *in vitro* stimulation, rendering them less susceptible to infection.[46,47] This would be in agreement with our observation of high CCR5 expression and lower capacity to produce β-chemokine *in vitro* in the CSW group.

In addition to CCR5 gene polymorphism, an analysis was conducted to determine the presence of the 64I substitution in CCR2b and of the 3′A genotype of SDF-1. As expected for an African study population, all our study subjects had a wild-type genotype for CCR5. In contrast, CCR2b and SDF-1 gene polymorphisms were present in the Ethiopian CSWs. The frequency of these mutations in the high-risk HIV-1-negative CSWs was comparable to what was found for other groups of Ethiopians.[38a] This suggests that polymorphisms in these genes are unlikely to play a role in HIV-1 infection susceptibility or resistance, confirming previous reports.[38,48]

In summary, our results show that coreceptor polymorphisms and expression levels, β-chemokine production, and cellular resistance to *in vitro* HIV-1 infection are not associated with protection in high-risk HIV-1-negative Ethiopian CSWs. Although PBMCs from CSWs could be infected *in vitro* equally as well as PBMCs from control women, higher proportions of controls produced higher amounts of p24 compared with the CSWs, indicating that postentry steps of the virus life cycle could be differentially regulated in these study groups. Indeed, several host cellular factors that regulate viral transcription have been described[49,50] that could influence viral replication levels and thereby establishment of infection *in vivo*. Other host mechanisms such as mucosal protective immunity,[7,9] HIV-1-specific cytotoxic T cell response,[8,26–28] and HLA type[24,25] have been indicated to be associated with resistance to HIV-1 infection. The role of these factors in the resistance of the high-risk Ethiopian CSWs remains to be investigated.

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