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Reduced HIV-1 Infectability of CD4$^+$ Lymphocytes from Exposed-Uninfected Individuals: Association with Low Expression of CCR5 and High Production of β-Chemokines

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We examined the human immunodeficiency virus type 1 infectability of CD4$^+$ lymphocytes isolated from CCR5 wild-type individuals, individuals heterozygous for the Δ32 allele of CCR5, and HIV-1-exposed but uninfected (EU) individuals who had CD4$^+$ lymphocytes refractory to M-tropic viral replication. None of the EU individuals were found to be heterozygous for the Δ32 allele. The CD4$^+$ lymphocytes isolated from CCR5/Δ32 and EU individuals were less infectable with an M-tropic viral isolate of HIV-1 than CCR5/CCR5 control individuals but were equally as infectable with a T-tropic viral isolate. The restriction to M-tropic viral isolate replication did not associate with any profound genotypic change in the CCR5 gene. CD4$^+$ lymphocytes from CCR5/Δ32 and CCR5/CCR5 EU individuals were more sensitive to the HIV-inhibitory effects of the recombinant β-chemokines RANTES, MIP-1α, and MIP-1β than were CD4$^+$ lymphocytes from CCR5/CCR5 control individuals. CD4$^+$ lymphocytes from EU individuals also showed increased sensitivity to recombinant β-chemokines and low surface expression of CCR5. A phenotype of low CCR5 expression and high secretion of β-chemokines is associated with reduced infectability of cells by M-tropic HIV-1 This phenotype may also be associated with protection against sexual transmission of HIV-1

INTRODUCTION

Various factors including viral phenotype, induced immune responses, and host genetic factors have been implicated in protection against transmission of the human immunodeficiency virus type 1 (HIV-1) (reviewed in Paxton and Koup, 1996). We previously reported on the relative resistance to *in vitro* infection of CD4$^+$ lymphocytes from a group of 25 multiply exposed individuals (Paxton *et al.,* 1996). Two of these individuals, EU2 and EU3, demonstrated a lack of infectability of their CD4$^+$ lymphocytes with macrophage-tropic nonsyncytium-inducing (M-tropic) isolates of HIV-1 but not with T-cell line-adapted syncytium-inducing (T-tropic) viral isolates (Connor *et al.,* 1996; Paxton *et al.,* 1996). Subsequent studies of these individuals revealed that they were both homozygous for a 32-bp deletion (Δ32) in the β-chemokine receptor CCR5, an HIV-1 coreceptor required for the entry of M-tropic viral isolates into macrophages, CD4$^+$ T lymphocytes, and microglial cells of the central nervous system (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Deng *et al.,* 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1996; He *et al.,* 1997; Liu *et al.,* 1996).

There is epidemiological evidence associating the Δ32 homozygous genotype with protection against the acquisition of HIV-1 (Dean *et al.,* 1996; Huang *et al.,* 1996; Samson *et al.,* 1996b; Zimmerman *et al.,* 1997). However, the recent identification of a few Δ32 homozygous individuals who have become infected with HIV-1 indicates that this protective effect is not absolute (Biti *et al.,* 1997; O'Brien *et al.,* 1997; Theodorou *et al.,* 1997). The majority of studies have indicated that Δ32 heterozygous individuals are not protected against HIV-1 transmission (Dean *et al.,* 1996; Huang *et al.,* 1996). HIV-1-infected individuals heterozygous for the Δ32 deletion have some advantage over CCR5 homozygote wild-type individuals in relation to disease progression (Dean *et al.,* 1996; Huang *et al.,* 1996; Zimmerman *et al.,* 1997). Heterozygotes are statistically more likely to harbor lower viral loads, have slower rates in CD4 lymphocyte decline, and live longer than CCR5 wild-type individuals (Dean *et al.,* 1996; Huang *et al.,* 1996; Samson *et al.,* 1996b; Zimmerman *et al.,* 1997). This protection is even stronger when only subjects infected with M-tropic viral isolates are analyzed (Michael *et al.,* 1997).

The mechanism by which heterozygous individuals are protected against disease progression is not fully understood. We have previously shown that CD4$^+$ lym-

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phocytes from heterozygous individuals are less infectable with M-tropic viral isolates of HIV-1 *in vitro* (Liu *et al.*, 1996). We also demonstrated that *in vitro* infectability correlated with CCR5 expression levels at the cell surface, as measured with a CCR5-specific monoclonal antibody (Wu *et al.,* 1997a). Lymphocytes isolated from Δ32 heterozygous individuals showed reduced CCR5 expression levels in comparison to CCR5 wild-type individuals, but there was substantial overlap in expression levels between the two groups of individuals.

Here we sought to address the mechanism by which some EU individuals have $CD4^+$ lymphocytes with a reduced capacity to replicate HIV-1 *in vitro* (Paxton *et al.,* 1996). We wished to address whether the observed reduction in *in vitro* viral replication was a result of other as yet unidentified polymorphisms within the CCR5 protein, the phenotype of CCR5 expression at the cell surface, or an increase in β-chemokine production levels.

RESULTS

CCR5 genotype analysis

We previously reported on the relative resistance of $CD4^+$ lymphocytes from a group of 25 highly exposed but uninfected individuals. Two of these individuals (EU2 and EU3) were homozygous for the 32-bp deletion in the CCR5 gene (Δ32/Δ32) (Liu *et al.,* 1996; Paxton and Koup, 1996). It had also been shown that two of the heterozygote (CCR5/Δ32) parents of EU2 and EU3 had reduced levels of M-tropic viral replication *in vitro,* resembling what had been observed for some of the EU individuals (Liu *et al.,* 1996). We therefore wished to genotype the remaining 23 individuals from the study group to determine whether the *in vitro* replication kinetics could be explained by an overrepresentation of heterozygotes. From the samples available we screened 21 individuals for the CCR5 genotype. One CCR5 Δ32/Δ32 individual was identified and no CCR5/Δ32 individuals, giving an overall frequency in the cohort of 13.0% Δ32/Δ32 (3/23), 87.0% (20/23) CCR5/CCR5 wild-types, and no CCR5/Δ32 individuals.

Infectability of $CD4^+$ lymphocytes from EU individuals

We used both M-tropic (JR-CSF) and T-tropic (NL4-3) isolates to infect $CD4^+$ enriched lymphocytes from 6 control CCR5 wild-type individuals (CCR5/CCR5), 4 Δ32 CCR5 heterozygote (CCR5/Δ32) controls, 2 Δ32 homozygotes (Δ32/Δ32) and 11 selected CCR5/CCR5 EU individuals. Due to the limited number of enriched $CD4^+$ lymphocytes not all individuals could be tested for NL4-3 infections. The EU samples tested were those which had previously shown lower levels of M-tropic viral replication (Paxton *et al.,* 1996). There were no statistical differences in the replication capacity of the T-tropic isolate between any of the four groups (Table 1), although a few of the EU individuals, specifically EU3 and EU12, had $CD4^+$ lymphocytes with low levels of T-tropic viral replication. However, when comparing the replicative capacity of the M-tropic viral isolate, differences were noted between the CCR5/CCR5 controls and CCR5/Δ32 controls ($P = 0.02$) and between CCR5/CCR5 controls and CCR5/CCR5 EUs ($P = 0.01$). These results demonstrate that the decreased replicative capacity in the CCR5/CCR5 EU $CD4^+$ lymphocytes is restricted to the M-tropic viral isolate JR-CSF and does not extend to the T-tropic isolate NL4-3. This suggests that the observed decrease in viral replication in the EU subjects is CCR5 related.

TABLE 1

CCR5 Genotype and HIV-1 Infectivity

Code	Genotype	p24 (pg/ml) JR-CSF	p24 (pg/ml) NL4-3
Controls			
LP9	CCR5/CCR5	333,340	162,924
LP10	CCR5/CCR5	522,395	54,258
LP12	CCR5/CCR5	22,929	28,654
LP18	CCR5/CCR5	380,535	158,658
LP14	CCR5/CCR5	373,140	nd
LW5	CCR5/CCR5	363,180	110,312
Mean		332,586	102,961
LP19	CCR5/Δ32	169,974	167,316
LW8	CCR5/Δ32	121,788	335,120
LW9	CCR5/Δ32	886	129,708
LW2	CCR5/Δ32	103	3,222
Mean		73,182	158,841
EUs			
EU2	Δ32/Δ32	0	57,006
EU3	Δ32/Δ32	0	12,194
EU5	Δ32/Δ32	0	nd
Mean		—	34,600
EU1	CCR5/CCR5	13,157	75,956
EU4	CCR5/CCR5	98,555	68,214
EU6	CCR5/CCR5	75,872	nd
EU8	CCR5/CCR5	311	55,906
EU11	CCR5/CCR5	140,116	54,832
EU12	CCR5/CCR5	186,138	4,356
EU13	CCR5/CCR5	1,627	nd
EU15	CCR5/CCR5	140,850	195,454
EU17	CCR5/CCR5	11,259	nd
EU18	CCR5/CCR5	4,551	nd
EU20	CCR5/CCR5	1,253	58,928
Mean		61,244	73,378

Note. Study individuals, CCR5 genotype, and HIV-1 infectivity. CCR5 genotype was determined by PCR from genomic DNA. Infectivity was performed on activated enriched $CD4^+$ lymphocytes. All cultures received the same inoculum of M-tropic or T-tropic virus and p24 production was measured on day 7; nd, not done.

CCR5 gene sequence

We reasoned that the low levels of M-tropic viral replication observed in the CCR5/Δ32 and CCR5/CCR5 EU

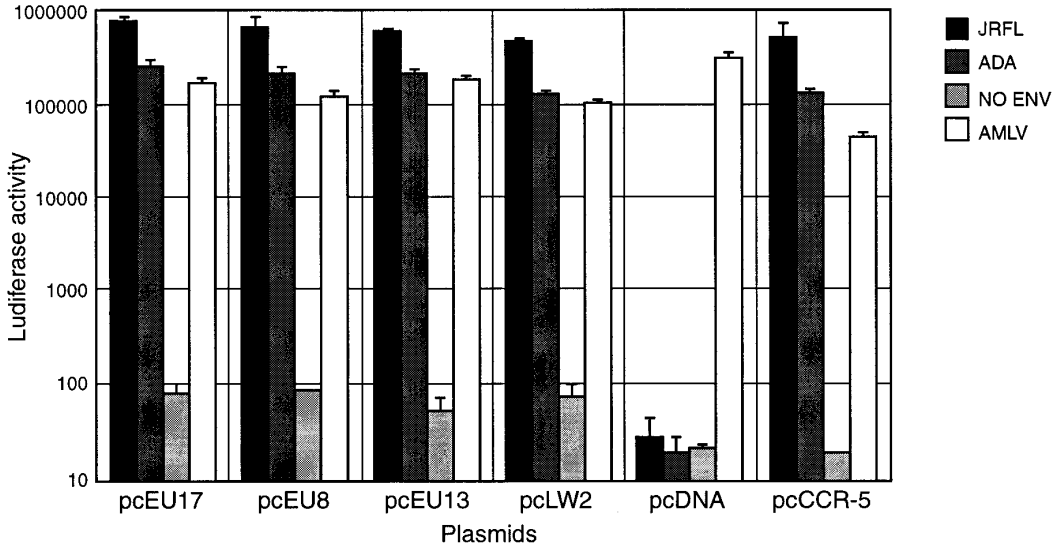

FIG. 1. Functional analysis of CCR5 clones from three EU individuals. 293T cells were cotransfected with pcDNAI/amp expressing CCR5 from EU individuals and pcCD4. Two days after transfection, these cells were infected with luciferase reporter viruses pseudotyped with the indicated macrophage-tropic (JR-FL, ADA) or A-MLV Env. Luciferase activity was measured 3 days after infection as described previously (Connor *et al.,* 1995; Deng *et al.,* 1996). The experiment was performed twice and for the EU individuals more than one clone was tested, with all giving similar results. Error bars indicated standard deviation of duplicate independent measurements.

$CD4^+$ lymphocytes resulted from either differential cell surface expression levels of the CCR5 protein or differences within the protein sequence which results in a CCR5 molecule with a reduced efficiency for M-tropic viral entry. To test the latter we amplified the full-length allele of CCR5 from genomic DNA of a CCR5/Δ32 individual (LW2) and both alleles from six of the CCR5/CCR5 EU individuals (EU6, 8, 12, 13, 17, and 18), all of whom had low levels of *in vitro* viral replication. These genomic amplicons were sequenced and were shown to be identical to the published CCR5 sequence (Samson *et al.,* 1996a). Several CCR5 clones were generated from each genomic amplicon. Sequencing of the individual EU CCR5 clones showed amino acid changes that did not represent individual alleles (data not shown). These changes are most likely to be PCR amplification or sequencing errors. To prove this, CCR5 from three EUs and LW2 were cloned into the expression vector pcDNAI/amp and were tested in a transient viral entry assay in which 293T cells were cotransfected with pcCD4 and CCR5 expression vectors and were infected with luciferase reporter viruses pseudotyped with M-tropic (JR-FL and ADA) or A-MLV env (Fig. 1). All of the EU CCR5 clones produced luciferase activity similar to that of the wild-type control, suggesting that they function normally in allowing M-tropic virus entry.

Sensitivity of $CD4^+$ lymphocytes to recombinant β-chemokines

We further characterized the *in vitro* infectability of $CD4^+$ lymphocytes from the various groups by analyzing their sensitivity to a cocktail of recombinant β-chemokines: RANTES, MIP-1α, and MIP-1β. The CCR5/Δ32 individuals had $CD4^+$ lymphocytes that were more sensitive to the HIV-1 inhibitory effects of the recombinant chemokines than $CD4^+$ lymphocytes from the CCR5/CCR5 control individuals (Fig. 2a). The $CD4^+$ lymphocytes from the CCR5/CCR5 EUs were also more sensitive to the recombinant chemokines than were the CCR5/CCR5 controls but not as sensitive as the CCR5/Δ32 cells (Fig. 2a). These differences are evident when comparing the ID_{50} and ID_{90} values for the recombinant proteins (Fig. 2b). The mean ID_{90} value for the CCR5/CCR5 control $CD4^+$ lymphocytes is 140.9 ± 33.1 ng/ml, with a range of 58.0–247.0 ng/ml; the CCR5/Δ32 cells have a mean ID_{90} value of 26.3 ± 12.4, with a range of 4.7–61.1 ng/ml, and the CCR5/CCR5 EUs have a mean ID_{90} value of 77.2 ± 26.0 ng/ml, with a range of 15.5–204. There was a statistical difference between the ID_{90} values for the CCR5/CCR5 control and CCR5/Δ32 individuals ($P = 0.03$) but not between the CCR5/CCR5 controls and CCR5/CCR5 EUs ($P = 0.15$), probably a result of the small sample size. There was, however, a trend toward increased sensitivity to β-chemokines among the CCR5/CCR5 EUs.

CCR5 surface expression levels

Given that the CCR5 DNA sequences from the CCR5/CCR5 EU individuals were wild-type and that isolated $CD4^+$ lymphocytes from these individuals were more sensitive, *in vitro,* to recombinant β-chemokines, we reasoned that these cells may be expressing lower cell surface levels of the CCR5 protein. We utilized monoclonal antibody 2D7 to measure CCR5 expression levels on

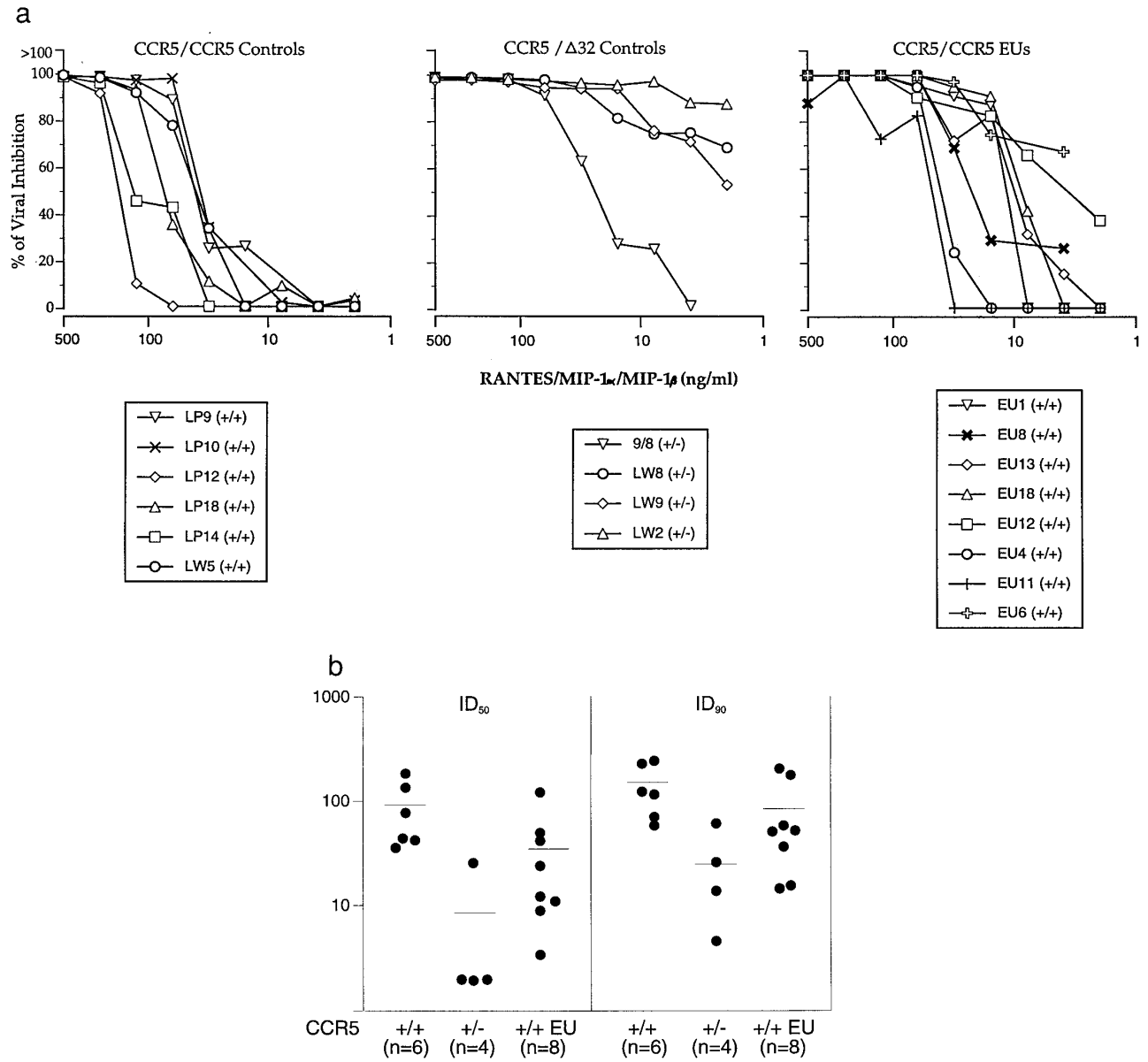

FIG. 2. Sensitivity of CD4+ enriched lymphocytes to recombinant β-chemokines. CD4+ enriched lymphocytes from CCR5/CCR5 control, CCR5/Δ32 control, or CCR5/CCR5 EUs were utilized in inhibition studies with variant (2× dilutions) of a cocktail of the recombinant chemokines RANTES, MIP-1α, and MIP-1β (starting concentration of 500 ng of each recombinant chemokine). The plots for percentage of viral inhibition in the presence of variant chemokine concentrations can be seen for each group in (a) and a plot of the calculated ID_{50} and ID_{90} values for each group can be seen in (b), with the mean values indicated as a bar.

unstimulated PBMCs from the study individuals (Wu *et al.,* 1997b). In addition, monoclonal antibody 12G5 was used to measure CXCR4 expression (Endres *et al.,* 1996). We used unstimulated PBMCs so as not to introduce error in expression levels due to *in vitro* stimulation. Monoclonal antibody 2D7 is able to stain cells in the absence of previous *in vitro* stimulation. The results of staining PBMCs from CCR5/CCR5 control, CCR5/Δ32 control, and CCR5/CCR5 EU individuals are shown in Fig. 3a. When comparing the three groups by mean fluorescence intensity (MFI) a statistical difference was observed between CCR5/CCR5 control and CCR5/CCR5 EU individuals ($P = 0.01$) for 2D7, but not 12G5 staining. The difference in 2D7 staining between the CCR5/CCR5 control and CCR5/Δ32 control individuals did not reach statistical significance ($P = 0.07$), most likely a reflection of the small number of CCR5/Δ32 individuals analyzed. We have previously shown in a larger group of subjects that there is a clear difference in surface expression of CCR5 between these groups (Wu *et al.,* 1997a). The two EU individuals, EU3 and EU12, with the CD4+ lymphocytes that demonstrated low infectability with a T-tropic viral isolate had 12G5 staining profiles similar to those of the other individuals.

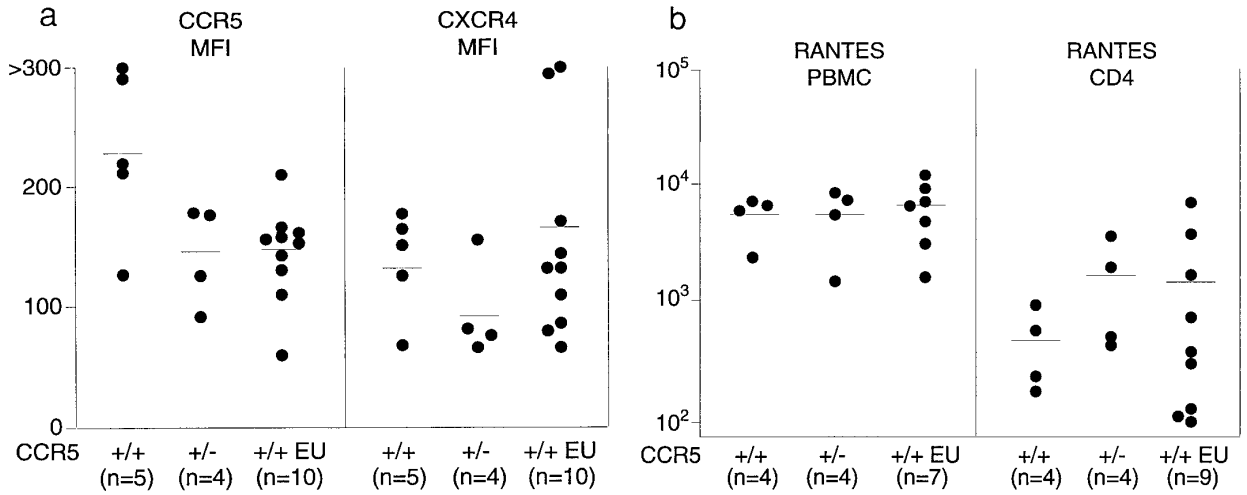

FIG. 3. Chemokine surface expression levels and β-chemokine secretion levels. CCR5 and CXCR4 cell surface expression levels were measured on unstimulated PBMCs and plotted as mean fluorescence intensity (MFI) for each group and can be seen in (a) with the mean value indicated by a bar. The RANTES levels (pg/ml) secreted from either PBMC fractions or $CD4^+$ enriched lymphocytes can be seen in (b), with the mean levels indicated by a bar.

A significant correlation was also noted between recombinant β-chemokine inhibition and CCR5 surface expression levels ($P < 0.05$, $r^2 = 0.26$) but there was no difference between ID_{50} values and CXCR4 surface expression levels. This suggests that levels of CCR5 surface expression directly affect the ability of β-chemokines to block M-tropic virus infection.

RANTES secretion levels

We also anticipated that high levels of endogenously produced β-chemokines might directly restrict HIV-1 entry and replication *in vitro.* Previously we had shown that enriched $CD4^+$ lymphocytes from two Δ32/Δ32 individuals secreted 5- to 10-fold higher levels of the β-chemokines RANTES, MIP-1α, and MIP-1β than $CD4^+$ lymphocytes from two CCR5/CCR5 control individuals (Paxton *et al.,* 1996). We measured the levels of RANTES being secreted from both PBMCs and enriched $CD4^+$ lymphocytes for representative individuals from all four groups. These results are shown in Fig. 3b. Although no statistical differences were found between any of the groups, the CD4 lymphocytes from a proportion of the CCR5/Δ32 control and CCR5/CCR5 EU individuals secreted higher levels of RANTES than the CCR5/CCR5 control individuals.

A statistical correlation was noted between RANTES secretion levels from enriched $CD4^+$ lymphocytes and recombinant β-chemokine inhibition (ID_{90}) levels ($P < 0.05$, $r^2 = 0.29$) but not between ID_{90} values and RANTES secretion levels from PBMCs.

DISCUSSION

The mechanism of decreased *in vitro* infectability of $CD4^+$ lymphocytes in EU individuals is not completely explained by the Δ32 CCR5 allele. From an original cohort of 25 EU individuals we selected 11 whose $CD4^+$ enriched lymphocytes demonstrated intermediate levels of M-tropic viral replication. We wished to address whether CCR5 genotype or phenotype was contributing to resistance to infection.

When the remainder of the individuals in the study cohort were genotyped for the Δ32 allele, one additional Δ32/Δ32 was identified (EU5) and no CCR5/Δ32 individuals were found. This provides an overall frequency in the cohort of 13.0% of Δ32/Δ32 individuals, supporting the correlation of this genotype with protection against the sexual transmission of HIV-1 (Dean *et al.,* 1996; Huang *et al.,* 1996; Samson *et al.,* 1996b; Zimmerman *et al.,* 1997). An interesting observation is that all three of the Δ32/Δ32 individuals belonged to the group of homosexual men who had reported sexual encounters with multiple partners (Paxton *et al.,* 1996). This high frequency (three of seven, 42.9%) in such a high-risk group further strengthens the premise that this genotype is overrepresented in the most highly exposed uninfected groups (Huang *et al.,* 1996). There have been recent reports describing Δ32/Δ32 individuals that are HIV-1 infected but none have yet described any Δ32/Δ32 individual as being specifically infected with an M-tropic, NSI, viral isolate (Biti *et al.,* 1997; O'Brien *et al.,* 1997; Theodorou *et al.,* 1997). No heterozygous individuals were identified in our EU population. The relative resistance of $CD4^+$ lymphocytes to M-tropic viral isolates within this cohort is therefore not a result of an overrepresentation of CCR5 Δ32 heterozygotes. We also conclude that the reduced replicative capacity of M-tropic viral isolates in the CCR5/CCR5 EU individuals is at the level of the CCR5 protein since $CD4^+$ lymphocytes from

these individuals were as readily infectable with the T-tropic strain NL4-3 as were the $CD4^+$ lymphocytes from CCR5/CCR5 control individuals.

The gene coding for CCR5 could be highly polymorphic; other alleles potentially exist which could alter the protein's capacity to function as an efficient coreceptor for HIV-1 entry. When tested directly, no DNA sequence variations were found in any of the CCR5 alleles from the selected individuals whose $CD4^+$ lymphocytes demonstrated the lowest levels of M-tropic viral replication, nor did the amplified clones show any reduced efficiency of HIV-1 entry into transfected 293/CD4 cells *in vitro* (Fig. 1). Therefore, the reduced infectability of $CD4^+$ lymphocytes from CCR5/CCR5 EU individuals was not due to previously unrecognized alleles within the CCR5 coding region.

Our study demonstrates that the $CD4^+$ lymphocytes from the CCR5/Δ32 and CCR5/CCR5 EU individuals are generally more sensitive to the effects of recombinant β-chemokines, have lower levels of CCR5 expression, and secrete higher levels of RANTES than do those of the CCR5/CCR5 control individuals. We found a twofold reduction in CCR5 cell surface expression levels in the CCR5/CCR5 EU individuals compared to controls. This level of expression is similar to what is commonly observed in CCR5/Δ32 individuals and could explain the reduced infectability of their $CD4^+$ lymphocytes to M-tropic virus (Table 1, Fig. 3a). In addition, the reduction in coreceptor expression occurs in the presence of higher endogenous secretion of RANTES, possibly leading to an enhanced antiviral effect. This combined effect is further demonstrated by the increased sensitivity of the $CD4^+$ lymphocytes from the CCR5/Δ32 and CCR5/CCR5 EU individuals to recombinant β-chemokines in comparison to CCR5/CCR5 controls. Our data are consistent with less β-chemokine being required to saturate the low level of CCR5 on the CCR5/Δ32 and CCR5/CCR5 EU cells, leading to more efficient blocking of the receptor from use by HIV-1. It is therefore likely that the combined effects of low surface expression of CCR5 and high secretion of β-chemokines are responsible for the lack of infection in many of our EU individuals. It should be noted that the donors with the least infectable $CD4^+$ lymphocytes had no one distinguishable characteristic. The low infectability with a T-tropic virus of $CD4^+$ lymphocytes from two EUs, EU3 and EU12, could not be explained by lower levels of CXCR4 expression at the cell surface.

On a more cautious note, we must be careful not to overinterpret these results. If we assume that a lowering of CCR5 cell surface expression levels has an effect on transmission of HIV-1, we would expect to find an increased frequency of CCR5/Δ32 individuals in the EU populations. Only one study to date has suggested that this genotype can protect against viral transmission (Samson *et al.,* 1996b); several other studies disagree with this conclusion (Dean *et al.,* 1996; Huang *et al.,* 1996; Zimmerman *et al.,* 1997). It is therefore likely that the increased production of β-chemokines, decreased surface expression levels of CCR5, and increased sensitivity to the antiviral effects of the β-chemokines among the CCR5/CCR5 EU subjects are only partially responsible for this *in vivo* protection against HIV-1 infection. Other factors are likely to be involved.

Our findings also have significance for the pathogenesis of HIV-1 infection. We and others have reported that the CCR5/Δ32 genotype protects against disease progression (Dean *et al.,* 1996; Huang *et al.,* 1996; Zimmerman *et al.,* 1997). Based upon the work presented here it is possible that lower levels of CCR5 and increased β-chemokine secretion levels from $CD4^+$ lymphocytes of some CCR5/CCR5 individuals may also protect against disease progression. In fact, CCR5 expression on PBMCs varies significantly between individuals (Wu *et al.,* 1997). We would predict that it is often the phenotype of CCR5 expression rather than the CCR5 genotype that is important in protection against disease progression. This phenotype may be linked to genotypes within CCR2b and SDF-1 that have recently been shown to protect against disease progression (Smith *et al.,* 1997; Winkler *et al.,* 1998).

The mechanism behind the low cell surface expression of CCR5 in CCR5/CCR5 individuals remains to be determined. There may be mutations within the promoter region of the gene or differences in transcriptional transactivation in these $CD4^+$ lymphocytes that alter the CCR5 mRNA levels. Additionally, there may be posttranscriptional events that account for the differences in CCR5 expression in $CD4^+$ lymphocytes between individuals. Further work in this area is needed. In the interim, measuring surface expression of CCR5 may provide important information into how coreceptor expression affects HIV-1 transmission and disease progression.

MATERIALS AND METHODS

Study individuals

The EU individuals were all taken from a previously described cohort of persons who had been exposed to HIV through multiple high-risk sexual encounters (Paxton *et al.,* 1996). The control individuals were either random Leukopac donors (LP) or laboratory workers (LW).

CCR5 genotyping

A portion of the CCR5 gene was amplified by PCR from genomic DNA (extracted via DNA/RNA isolation kits, USB, Cleveland, OH) and analyzed on a 4% Metaphor agarose gel. The primers CCR5c, 5′-CAAAAAGAAGGTCTTCATTACACC-3′, and CCR5d, 5′-CCTGTGCCTCTTCTTCTCATTTCG-3′, that flank the 32-bp deletion were used to generate wild-type and deleted fragments of 189

and 157 bp, respectively. The PCR reaction mixture contained 0.25 mM dNTPs, 20 pmol of each primer, and 0.5 unit of *Taq* polymerase in 1× reaction buffer (Promega, Madison, WI). Each PCR amplification consisted of 42 cycles, with the first 5 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 45 s, followed by 37 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s.

Cloning and sequencing of the CCR5 gene

The CCR5 gene was amplified from genomic DNA using primers flanking the coding region, 5′-CTCGGATCCGGTGGAACAAGATGGATTAT, 5′-CTCGTCGACATGTGCACAACTCTGACTG, with Expand polymerase (Boehringer Mannheim) by 5 cycles of PCR (94°C, 30 s; 55°C, 45 s; 72°C, 90 s) followed by an additional 35 cycles (94°C, 30 s; 62°C, 45 s; 72°C, 90 s) in 100 μl. Only the full-length allele was amplified from heterozygous (CCR5/Δ32) genomic DNA using a pair of overlapping primers that would hybridize to the deleted 32 bp, 5′-GTCTGGAAATTCTTCCAGAATTGATAC, 5′-GTATCAATTCTGGAAGAATTTCC. The PCR products were either sequenced directly or cloned into the expression vector pcDNA/amp (Invitrogene Corp.) and then sequenced using either the dideoxy method or high-density oligonucleotide arrays (Kozal *et al.,* 1996).

CD4 infectability and recombinant chemokine sensitivities

All cells utilized in this study were isolated from previously cryopreserved peripheral blood mononuclear cells (PBMC). Cells were thawed and stimulated with PHA (5 μg/ml) and carried in culture medium (RPMI 1640 supplemented with antibiotics and 10% fetal bovine serum) containing interleukin-2 (100 units/ml) (Hoffman–LaRoche, Nutley, NJ). The cells were carried for 7 days. Half the medium was replaced with fresh culture medium containing IL-2 every 2–3 days. On day 7 the cells were enriched for $CD4^+$ lymphocytes by depleting the culture of the $CD8^+$ cells using immunomagnetic beads (DYNAL, Great Neck, NJ). Viral infections were set up using 2.0×10^5 $CD4^+$ enriched lymphocytes per well of flat-bottomed 96-well plates with a 3000 median tissue culture infectious dose ($TCID_{50}$) of the M-tropic isolate JR-CSF and separately the T-tropic virus pNL4-3 (m. o. i. = 0.003). Cells were cultured for 7 days and fed on day 4 with fresh interleukin-2 containing media. Viral growth was determined on day 7 by measuring p24 antigen levels using an immunoassay (Abbott Laboratories, Abbott Park, IL). The sensitivity of the $CD4^+$ enriched lymphocytes to β-chemokines was assessed by incubating 1.0×10^5 of the above-described cells (100 μl/well in flat bottomed 96-well plates) with varying quantities of the recombinant chemokines RANTES, MIP-1α, and MIP-1β (R & D Systems, Minneapolis, MN). All three chemokines were mixed in a cocktail with a starting concentration of 1000 ng/ml of each recombinant chemokine. Twofold dilutions of this initial cocktail were made down to 3.9 ng/ml, and 100 μl of each was added to separate wells of $CD4^+$ enriched lymphocytes. A control well containing media alone was included in all assays. Each well was inoculated with 3000 $TCID_{50}$ of the M-tropic viral isolate JR-CSF. The cultures were carried for 7 days and fed with fresh interleukin-2 containing media on day 4. Viral activity was measured as described above on day 7 and viral inhibition determined as the percentage of p24 reduction, in the presence of recombinant chemokine, compared to the control well. The chemokine concentrations required to inhibit p24 production by 50 or 90% (ID_{50} and ID_{90} values) were calculated using linear regression analysis. The results presented were from single experiments that were reproducible.

RANTES secretion levels

Seven-day activated PBMCs or $CD4^+$ enriched lymphocytes were plated at 1.0×10^6/well in a 24-well plate and cultured for a further 4 days. The supernatants were harvested and RANTES levels determined using a commercially available immunoassay (R & D Systems). The results presented were from single experiments that were reproducible.

CCR5 and CXCR4 surface expression levels

FACS analysis was performed on unstimulated PMBCs using the 2D7 (Wu *et al.,* 1997b) and 12G5 (Endres *et al.,* 1996) monoclonal antibodies to determine CCR5 and CXCR4 expression levels at the cell surface, respectively. The 2D7 and 12G5 antibodies were used as purified antibody and utilized at a working concentration of 8 and 1.7 μg/ml, respectively. The antibodies were incubated with 2.0×10^5 cells in 50 μl PBS/5%FCS/0.01% sodium azide solution for 30 min on ice. Isotype-matched mouse monoclonal antibodies were used as controls (IgG1 for 2D7 and IgG2a for 12G5, Caltag, San Francisco, CA). Cells were washed three times with PBS containing 5% FCS and 0.01% sodium azide before being incubated with a 1:50 dilution of a RPE-conjugated goat anti-mouse antibody (DAKO Inc., Capinteria, CA). The cells were incubated for 30 min on ice and again washed three times before being fixed in 1% formaldehyde. Surface staining was analyzed with a FACS calibur machine (Becton–Dickinson, San Jose, CA).

Statistical analysis

The unpaired Student's *t* test was used to compare similar parameters between two separate groups of individuals. Regression analysis was used when comparing two different parameters. In both tests *P* values of <0.05 were considered significant.

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