

HIV-1-resistance phenotype conferred by combination of two separate inherited mutations of *CCR5* gene

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Summary

Background Despite multiple exposures to HIV-1, some individuals remain uninfected, and their peripheral-blood mononuclear cells (PBMC) are resistant to in-vitro infection by primary HIV-1 isolates. Such resistance has been associated with a homozygous 32-base-pair deletion ($\Delta 32$) in the C-C chemokine receptor gene *CCR5*. We examined other mutations of the *CCR5* gene that could be associated with resistance to HIV-1 infection.

Methods We assessed the susceptibility of PBMC to in-vitro infection by HIV-1 isolates that use the *CCR5* as the major coreceptor for viral entry in 18 men who had frequent unprotected sexual intercourse with a seropositive partner. We also did genotypic analysis of *CCR5* alleles. One of the 18 exposed but uninfected men (who we refer to as ExU2) showed total resistance to in-vitro infection by *CCR5*-dependent viruses, and was found to carry a *CCR5* $\Delta 32$ allele and a single point mutation (T→A) at position 303 on the other allele. To find out whether the *CCR5* mutation was restricted to ExU2's family or existed in the general population, we did genetic analyses of the *CCR5* genotype in ExU2's father and sister and also in 209 healthy blood donors who were not exposed to HIV-1.

Findings The m303 mutation found in ExU2 introduced a premature stop codon and prevented the expression of a functional coreceptor. The family studies revealed that the m303 mutant allele was inherited as a single mendelian trait. Genotype analysis showed that three of the 209 healthy blood donors were heterozygous for the mutant allele.

Interpretation We characterise a new *CCR5* gene mutation, present in the general population, that prevents expression of functional coreceptors from the abnormal allele and confers resistance to HIV-1 infection when associated to the $\Delta 32$ *CCR5* mutant gene.

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Introduction

Some individuals remain uninfected by HIV-1 despite multiple sexual exposures to the virus. The peripheral-blood mononuclear cells (PBMC) of such individuals are resistant to in-vitro infection by strains of HIV-1 that use the C-C chemokine receptor *CCR5* as the major coreceptor. This resistance is associated with a homozygous 32-base-pair deletion ($\Delta 32$) in the *CCR5* gene,^{1,2} and only a few cases of HIV-1 infection among individuals homozygous for *CCR5* $\Delta 32$ have been reported.³⁻⁵ Individuals heterozygous for this deletion are usually susceptible to infection,⁶ and their PBMC are permissive to *CCR5*-dependent HIV-1 strains.¹ *CCR5* is the main coreceptor of the HIV-1 strains that are preferentially involved in sexual transmission⁷⁻¹⁰ and are more frequently detectable in newly infected individuals.¹¹ By contrast, HIV-1 strains that appear later in the course of the disease use other coreceptors, such as CXCR4.^{12,13} *CCR5* $\Delta 32$ homozygosity occurs in about 1% of the white population.¹ In groups at high risk of HIV-1 infection, the frequency is only slightly higher (2.8%),⁶ which suggests that other non-genetic factors could account for resistance to infection. On the other hand, alterations of the *CCR5* gene other than the $\Delta 32$ allele could be associated with the HIV-1 resistance phenotype. We looked at the susceptibility of PBMC from homosexual men who had frequent and regular unprotected sexual intercourse with a seropositive partner to in-vitro infection by HIV-1 isolates with *CCR5* or CXCR4 as the main coreceptors for viral entry.

Methods

We collected blood samples from 18 men who were not infected with HIV-1, but who had frequent unprotected sexual intercourse with their HIV-1-infected partners for at least 2 years. Our inclusion criteria required each participant to be negative for the presence of specific HIV-1 antibodies (ELISA) and HIV-1 RNA (HIV-1 RNA Monitor Roche, Roche Diagnostic Systems, Branchburg, NJ, USA). All participants were informed of the purpose of the study and agreed to take part, they were also counselled for safe-sex practice. The study protocol was approved by the local ethics committee. We also tested blood samples from an already established cohort of 209 blood donors for genetic analyses of the *CCR5* genotype.

Phytohaemagglutinin-A-activated Ficoll-purified human PBMC were infected with supernatants from HeLa cells transfected with YU2 (*CCR5* dependent) or NL4-3 (CXCR4 dependent) DNA HIV-1 proviruses. Two million activated PBMC were infected with HIV-1: the supernatant contained 10 ng p24 HIV-1 antigen, measured by ELISA (HIV-1 p24 Core Profile ELISA, Dupont De Nemours, Wilmington, DL, USA). Cells were washed three times with phosphate-buffered saline and cultured at 10⁶ cells/mL in RPMI 1640 medium (Gibco BRL Life Technologies, Gaithersburg, MD, USA) which contained 10% fetal calf serum and 20 ng/mL recombinant interleukin-2 (EuroCetus, Amsterdam, Netherlands). Every 3 days, the culture fluid was harvested and replaced with fresh medium that contained

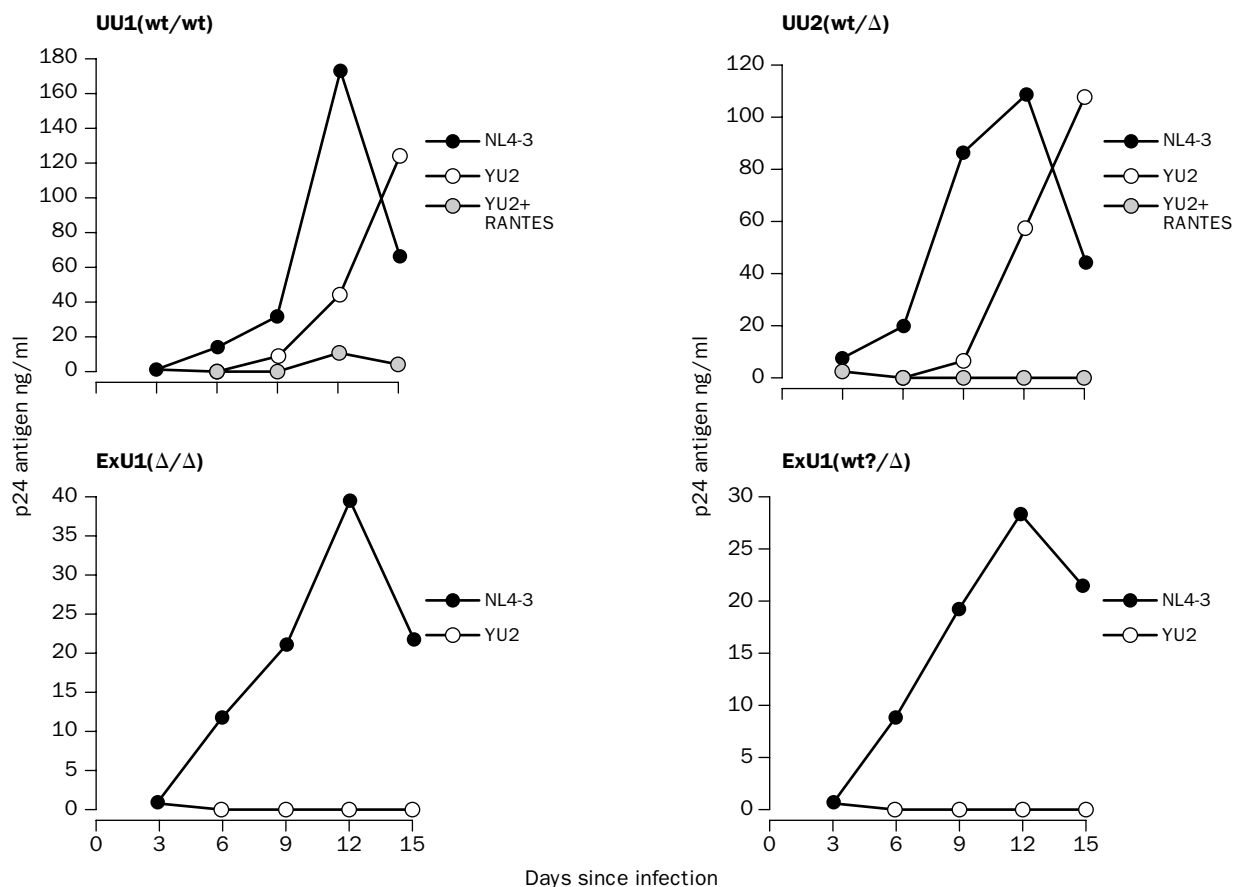


Figure 1: **HIV-1 infection of PBMC from two unexposed uninfected men (UU1 and UU2) and two exposed and uninfected men (ExU2 and ExU1)**
CCR5 genotype for *CCR5* $\Delta 32$ deletion indicated in parentheses: (wt/wt): homozygous wild type; (wt/ Δ): heterozygous $\Delta 32$; (Δ/Δ): homozygous $\Delta 32$; (wt? Δ): heterozygous $\Delta 32$ with an apparent wild-type allele.

recombinant interleukin-2, and the amount of p24 antigen in the cell-free supernatants was measured. The recombinant human chemokine RANTES (Biodesign International, Kennebunk, ME, USA) was added at 125 nmol/L final concentration at the time of infection and each time the medium was replaced.

For the assessment of HIV-1-coreceptor function of *CCR5* mutants, we used an HIV-1 envelope-glycoprotein-mediated cell-fusion assay. HeLa CD4 cells (clone p4), which express human *CD4* gene, and a *lacZ* reporter gene, driven by the HIV-1 long-terminal-repeat (LTR) promoter,¹⁴ were transiently transfected with a pcDNA3 vector that expresses *CCR5* wild type, *CCR5* $\Delta 32$, or *CCR5* m303 DNA. HeLa CD4 cells (2×10^5) transfected with *CCR5* derivatives were cocultured for 24 h with HeLa cells that express the HIV-1 transactivator Tat and the envelope glycoprotein of HIV-1 LAI (HeLa LAI)¹⁵ or HIV-1 Ada (HeLa Ada)¹⁶. We used a HeLa cell clone that expresses Tat but does not express envelope HeLa as a control in the cell-fusion assay.¹⁵ Fusion of the cocultured cells results in cytoplasmic mixing and induction of the HIV-1 LTR-driven *lacZ* gene by the Tat protein. β -galactosidase activity expressed from the *lacZ* reporter gene was assessed in cell lysates with a colorimetric assay.¹⁷

For detection of the *CCR5* $\Delta 32$ deletion, we amplified the specific gene fragment by PCR from PBMC genomic DNA with two primers: 5'-GTCTTCATTACCTGCAGCTC-3' and 5'-GTGAAGATAAGCCTCACAGCC-3'. PCR was done with 1 μ g genomic DNA as previously described by Samson and colleagues.¹ PCR products were separated by agarose-gel electrophoresis. We obtained two fragments of 198 base pairs and 166 base pairs that corresponded to the wild type and the deleted *CCR5* alleles. Genomic *CCR5* DNA was amplified by PCR with primer 5'-CCCAAGCTTATGGATTATCAA-3' and

primer 5'-GCTCTAGATCACAAGCCCACAGA-3'. PCR was done with 1 μ g genomic DNA or 200 ng plasmid DNA with 0.2 mmol/L dNTPs, 0.2 μ mol/L primers, and 1.25 U AmpliTaq Gold for 30 cycles (94°C, 1.5 min; 47°C, 1 min; 72°C, 1.5 min), after an initial 10 min denaturation at 94°C. The amplified products were cloned in a pCR3 vector with the TA cloning kit (Invitrogen, Leek, Netherlands) and used for enzymatic-restriction analysis and nucleotide sequencing of the two DNA strands by cycle-sequencing and dye-terminator methods with an automated DNA Sequencer ABI 377 (PE Applied BioSystems, Foster City, CA, USA).

One of the 18 men (who we refer to as ExU2) showed total resistance to in-vitro infection by *CCR5*-dependent viruses. He was heterozygous for the $\Delta 32$ *CCR5* allele and remained uninfected despite frequent unprotected sexual intercourse with several HIV-1-infected partners between 1986 and 1996.

The resistance of ExU2's PBMC to in-vitro infection by *CCR5*-dependent viruses, together with his healthy clinical status despite a history of sexual exposure to HIV-1, prompted us to look for an alternative mutation that rendered inactive the non-deleted *CCR5* allele. We, therefore, cloned the entire *CCR5* gene of ExU2 and determined the nucleotide sequence of the two alleles.

We also investigated whether the *CCR5* mutation was a new mutation that had developed in ExU2 or was inherited as a mendelian trait. We, therefore, did *CCR5* genetic analysis and PMBC infection assays on ExU2's father and sister with their informed consent. To assess whether the *CCR5* mutation was restricted to the family of ExU2 or was also present in the general population, we tested genomic DNA from the 209 healthy blood donors by restriction-enzyme analysis.

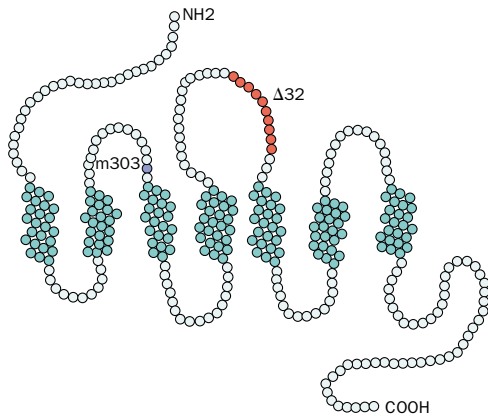


Figure 2: **Amino acid sequence of CCR5**

Position of m303 mutation and $\Delta 32$ deletion are shown.

We tested the in-vitro susceptibility of ExU2's PBMC to infection by two molecular clones of HIV-1, YU2¹⁸ and NL4-3,¹⁹ which use CCR5 or CXCR4, respectively, as coreceptors.¹⁷ We then compared the susceptibility of ExU2 to HIV-1 infection with that of another exposed but uninfected man in the group who was homozygous for the $\Delta 32$ CCR5 allele (ExU1) and also with that of two healthy blood donors who were not infected with HIV-1 and were not exposed to the virus—one (UU1) carried the wild-type allele (wt/wt) and the second (UU2) was heterozygous for the deleted allele (wt/ Δ).

To show that the resistance of ExU2's PBMC to infection by CCR5-dependent viruses was not restricted to the YU2 molecular clone, we infected PBMC from ExU2 and UU2 with two additional CCR5-dependent HIV-1 isolates (JRCSF and BaL). We also investigated whether PBMC from ExU2 were resistant to infection by the HIV-1 isolate V164, which we obtained from the seropositive partner of ExU2. We have previously characterised the V164 virus isolate as CCR5 dependent on the basis of its susceptibility to RANTES and its inability to grow on MT2 and C8166 cells.

Results

Figure 1 shows HIV-1 infection of PBMC from UU1 and UU2 (the two unexposed uninfected individuals), and from ExU2 and ExU1 (the men who were exposed to HIV-1 but remained uninfected) with CCR5-dependent (YU2) and CXCR4-dependent (NL4-3) HIV-1 molecular clones, with or without addition of RANTES. The results are expressed as the amount of p24 antigen in culture supernatants, and represent four independent experiments. PBMC from all four individuals were readily infected by NL4-3. By contrast, PBMC from ExU1 and ExU2 were resistant to infection by YU2, whereas UU1 and UU2 were susceptible to infection. The same results were obtained with CD8-cell-depleted PBMC (data not shown). Infection with YU2 in PBMC from UU1 and UU2 was inhibited by RANTES, a ligand of CCR5 which prevents viral entry of a large series of primary HIV-1 isolates.²⁰ We also found that PBMC from ExU2 were refractory to infection by two additional CCR5-dependent HIV-1 isolates as well as the clinical isolate V164. By contrast, PBMC from UU2 were susceptible to infection by all viruses tested (data not shown).

Cloning of the entire CCR5 gene of ExU2 and nucleotide sequencing of each allele confirmed the presence of the $\Delta 32$ deletion in one of the two alleles. In addition, a single point mutation (T→A) at position 303 was found in the non-deleted allele (m303; figure 2). The rest of the sequence was identical to the wild-type gene.²¹

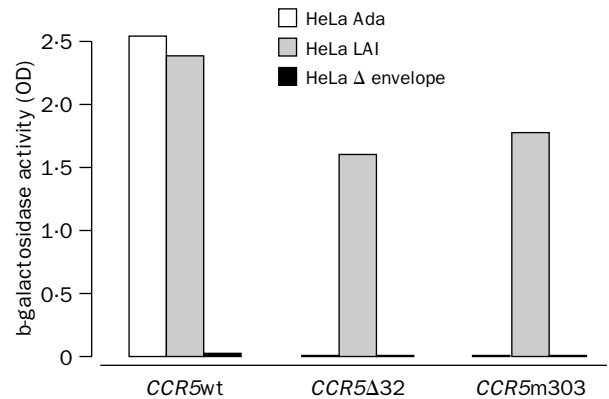


Figure 3: **Envelope-mediated cell-fusion assay for analysis of CCR5 from UU1, UU2, ExU2, and ExU1**

p4 cells transfected with CCR5 wild type (CCR5wt), m303 (CCR5 m303), or $\Delta 32$ (CCR5 $\Delta 32$). DNA were cocultured with HeLa cells that express HIV Ada or LAI Env. Cells without Env (HeLa Δ Env) were used as control. Cell fusion was evaluated after 24 h by measurement of β -galactosidase activity (the SE is less than 5%).

The m303 mutation generates a premature stop codon in the CCR5 gene, which implicates the loss of a unique restriction-enzyme site (HincII). The capacity of the m303 CCR5 product to function as an HIV-1 coreceptor was assessed by HIV-1 an envelope-mediated cell-fusion assay which reflects the ability of HIV-1 to enter a cell.^{7-9,12,13}

Figure 3 shows that the CCR5 wild-type allele was the only one able to generate a functional—ie, fusion-permissive coreceptor—when the fusion assay was done with cells that express the CCR5-dependent envelope HIV-1 Ada. By contrast, both the $\Delta 32$ and the m303 mutant allele products did not support cell fusion. As expected, HeLa LAI cells fused with all the HeLa transfected cells because CXCR4 is constitutively expressed by HeLa cells. These results suggest that ExU2's resistance to infection by primary CCR5-dependent HIV isolates was due to the absence of a functional CCR5 coreceptor.

Figure 4 shows the loss of a HincII restriction site in the CCR5 m303 allele. This restriction-enzyme site allowed us to carry out a rapid screening of genomic PCR-amplified DNA that corresponded to ExU2, his sister and father, ExU1, UU1, and UU2. ExU2's father and sister were heterozygous for the $\Delta 32$ deletion (figure 5). Digestion with the restriction enzyme HincII generated different restriction patterns in the CCR5 alleles. Thus, we obtained two distinct DNA fragments of apparent molecular size of 0.7 kb and 0.3 kb from the individuals who carried the wild-type genotype (UU1) or the $\Delta 32$ allele as a homozygous (ExU1) or heterozygous (UU2) trait (figures 6, 7, lanes 4, 5, and 6). In addition to the two DNA fragments, the enzymatic restriction pattern of ExU2 and his sister showed an additional 1.1 kb DNA fragment (figure 7, lanes 1 and 2) that

							312
N	T	M	C	Q	L	L	
aat	aca	atg	tgt	caa	ctc	ttg...	wt
							HincII
N	T	M	Z	Q	L	L	
aat	aca	atg	tga	caa	ctc	ttg...	m303
							Absence of HincII

Figure 4: **DNA nucleotide sequences of wild type (wt) and m303 alleles**

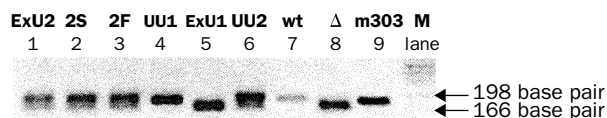


Figure 5: PCR amplification of genomic DNA by specific primers for the $\Delta 32$ deletion

A 1 kb DNA ladder was used as a marker (M). Genomic DNA after $\Delta 32$ PCR analysis is designated as: ExU2=exposed uninfected (m303/ $\Delta 32$), 2S=sister of ExU2 (wt?/ Δ), 2F=father of ExU2 (wt?/ Δ), UU1=unexposed uninfected (wt/wt), ExU1=exposed uninfected ($\Delta 32/\Delta 32$), UU2=unexposed uninfected (wt/ Δ). Plasmid DNA corresponds to wild type (wt), $\Delta 32$, and m303 cloned alleles.

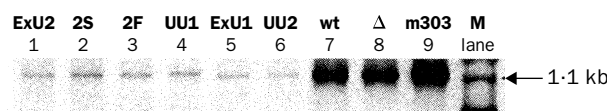


Figure 6: PCR amplification of the entire *CCR5* gene from genomic (lanes 1–6) and plasmid (lanes 7–9) DNA

Abbreviations and explanations as in figure 5.

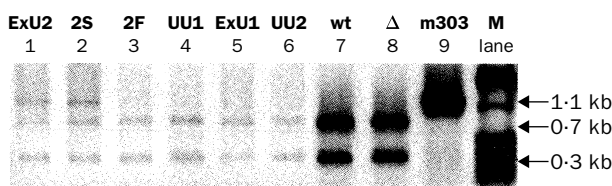


Figure 7: HincII enzymatic digestion of PCR amplified DNA (lanes 1–6) or the indicated *CCR5* alleles after cloning in pCR3-1 (lanes 7–9)

Abbreviations and explanations as in figure 5.

corresponded to the mutated *CCR5* allele without HincII. Results were identical when we did the restriction enzyme analysis with *CCR5* wt, *CCR5* $\Delta 32$, and *CCR5* m303 DNA cloned in a pCR3-1 plasmid (figure 7, lanes 7–9). Thus, ExU2 and his sister share the same *CCR5* genotype, which suggests that the *CCR5* m303 allele was inherited from their mother. We were not able to obtain definitive evidence of this inherited allele because their mother died long before our study took place. Both the 32-base-pair deletion and m303 mutation in ExU2's sister's alleles were confirmed by nucleotide sequencing (data not shown).

Since ExU2 and his sister carry the same *CCR5* genetic trait, we investigated the in-vitro susceptibility of their PBMC to infection with YU2, the *CCR5*-dependent virus, and compared it with that of PBMC from their father (wt/ $\Delta 32$ genotype). As we expected, based on the lack of function of the two mutant alleles, PBMC from the sister displayed the same degree of resistance to infection as PBMC from ExU2, whereas PBMC from the father were fully susceptible to infection with YU2 (figure 8). All three family members were susceptible to in-vitro infection with NL4-3, the CXCR4-dependent virus (figure 8). Thus, the combination of two independent *CCR5* genetic defects confers in-vitro resistance to infection by *CCR5*-dependent viruses.

Our tests of genomic DNA from the 209 healthy blood donors who were not exposed to HIV-1 showed that three individuals carried an m303 mutant allele, as identified by HincII-restriction-enzyme analysis (none were m303/ Δ). We confirmed the presence of the T→A point substitution at nucleotide 303 by nucleotide sequencing of PCR-amplified DNA in all three individuals.

Discussion

Our findings document the existence of a *CCR5* gene anomaly—other than the previously identified $\Delta 32$ base-pair deletion—that prevents the expression of a functional HIV-1 coreceptor from the affected allele. The T→A point mutation at nucleotide 303 introduces an early stop codon in the open reading frame of the gene. The inability of the mutated allele to support cell–cell fusion, mediated by envelope glycoproteins from HIV-1 primary isolates, indicates that the final product of the *CCR5* m303 mutant is a truncated protein without any coreceptor function. The m303 mutant allele which we identified in ExU2, who has been at high risk of exposure to HIV-1 during the past 10 years, proved to be inherited as a single mendelian trait. In addition, our genetic analysis of the healthy blood donors revealed that the m303 mutant allele is not a sporadic point mutation in an isolated family but is also present in the general population. Although the existence of other *CCR5* gene alterations has been reported,²² there is no experimental evidence that *CCR5* anomalies, other than the homozygous 32-base-pair deletion, are associated with resistance to HIV-1 infection. By contrast, the *CCR5* point mutation that we document, when associated with the 32-base-pair deletion, renders PBMC resistant to HIV-1 infection by primary isolates (the predominant forms of the virus during the early stages of infection). The combination of two distinct *CCR5* gene alterations (*CCR5* $\Delta 32$ /m303) in ExU2 and his sister who was not exposed to HIV-1 confers a resistance phenotype to HIV-1 infection in vitro, which is indistinguishable from that of the $\Delta 32$ homozygosity. The lack of susceptibility to infection by primary isolates shown by PBMC from ExU2 and his sister, on the one hand, and their

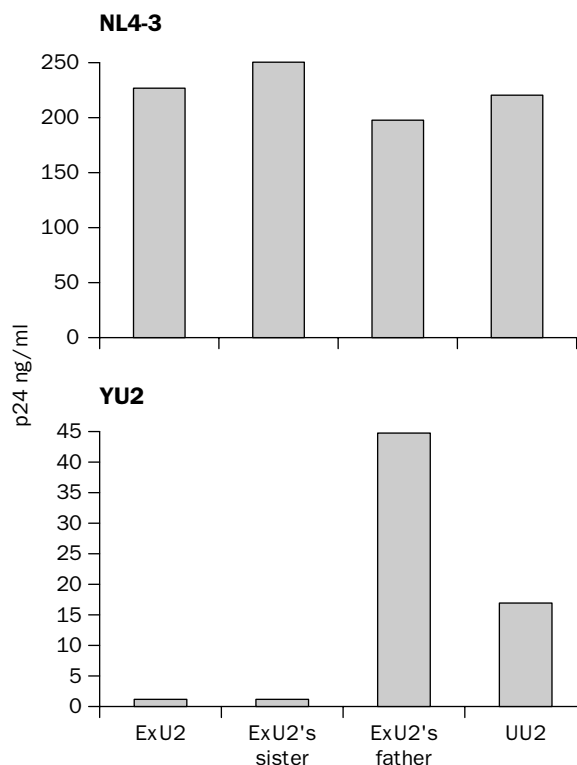


Figure 8: Infection of PBMC from ExU2, his sister and father, and UU2 with 0.5 ng p24/10⁶ cells of YU2 or NL4-3 HIV-1 molecular clones

Results are amount of p24 antigen in cell-free culture supernatants.

susceptibility to infection by CXCR4-dependent viral isolates on the other, suggest that the simultaneous presence of two defective *CCR5* alleles renders cells non-permissive to primary isolates without interfering with the capacity of the target cells to replicate HIV-1 (infection and replication of CXCR4-dependent viruses).

The abnormal *CCR5* genotype of ExU2 probably reflects his remarkable resistance to sexual acquisition of HIV-1 infection, which is also indicated by the fact that his PBMC were non-permissive to the virus (V164) isolated from his HIV-1-infected partner with whom he has regular and unprotected sexual intercourse.

We were not able to identify the precise frequency of the *CCR5* m303 mutant allele because we did not take account of the ethnic origin of the 209 healthy HIV-1-unexposed individuals. But the presence of the m303 allele in three of these individuals suggests that the frequency in western European countries is appreciable. Thus, putative m303 homozygosity and its association with the 32-base-pair deleted allele could account for unexplained cases of HIV-1 resistance in individuals who carry an apparent wild-type *CCR5* genotype or a $\Delta 32$ heterozygous trait. Moreover, the apparent increased resistance to HIV-1 infection of *CCR5* $\Delta 32$ heterozygous individuals,^{1,6,23-25} and the low frequency of this allele among HIV-1-infected people who rapidly progress to disease^{6,23-25} suggests that similar effects could account for the presence of an m303 mutation as a heterozygous trait.

Contributors

Alberto Beretta and Luc Montagnier initiated the project. Alberto Beretta and Fernando Arenzana-Seisdedos were responsible for the experimental design and analysis of data. Caroline Quillent conducted some of the virus-infection assays, developed the restriction-enzyme analysis of the *CCR5* alleles, and conducted, together with Patricia Métais, the genetic analysis of the healthy blood donors. Estelle Oberlin and Dominique Rousset prepared supernatants of cloned HIV-1 viruses, did some of the virus-infection assays and the cell-fusion assays with *CCR5*-transfected cell lines. Joséphine Braun generated and tested the clinical HIV-1 isolate, and also carried out some of the infection assays. Gustavo Gonzalez Canali was responsible for clinical matters. Jean-Louis Virelizier was involved in writing the paper and suggested critical experiments. All the authors read and contributed to the writing of the paper.

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