

A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promoter mutation

LEONDIOS G. KOSTRIKIS¹, YAOXING HUANG¹, JOHN P. MOORE¹, STEVE M. WOLINSKY²,
LINQI ZHANG¹, YONG GUO¹, LISA DEUTSCH³, JOHN PHAIR²,
AVIDAN U. NEUMANN³ & DAVID D. HO¹

¹The Aaron Diamond AIDS Research Center, The Rockefeller University, 455 First Avenue,
New York, New York 10016, USA

²Department of Medicine, Northwestern University Medical School, 303 East Chicago Avenue,
Chicago, Illinois 60611, USA

³The Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel

Correspondence should be addressed to D.D.H.

Viral and host factors influence the rate of HIV-1 disease progression¹. For HIV-1 to fuse, a CD4⁺ cell must express a co-receptor that the virus can use^{2,3}. The chemokine receptors CCR5 and CXCR4 are used by R5 and X4 viruses, respectively⁴. Most new infections involve transmission of R5 viruses, but variants can arise later that also use CXCR4 (R5-X4 or X4 viruses)²⁻⁶. This is associated with an increased rate of CD4⁺ T-cell loss and poor prognosis²⁻⁶. The ability of host cells to support HIV-1 entry also influences progression. The absence of CCR5 in approximately 1% of the Caucasian population, due to homozygosity for a 32-nucleotide deletion in the coding region (Δ 32-CCR5 allele), very strongly protects against HIV-1 transmission⁷⁻¹⁰. Heterozygosity for the Δ 32-CCR5 allele delays progression typically by 2 years^{9,10}. A recent study showed that a conservative substitution (V64I) in the coding region of CCR2 also has a significant impact on disease progression, but not on HIV-1 transmission¹¹. This was unexpected, since CCR2 is rarely used as a co-receptor *in vitro*^{2,3} and the V64I change is in a transmembrane region¹¹. Because a subsequent study did not confirm this effect on progression to disease¹², we analyzed CCR2-V64I using subjects in the Chicago MACS. We show that CCR2-V64I is indeed protective against disease progression and go on to show that the CCR2-V64I allele is in complete linkage disequilibrium with a point mutation in the CCR5 regulatory region.

Rapid epidemiological studies on the Δ 32-CCR5 allele were possible because the truncated DNA species could be readily detected by gel electrophoresis after PCR amplification of the coding region. The CCR2-64I and wild-type alleles do not differ in length, however. They can be distinguished by sequencing, but this is unwieldy. Single-strand conformation polymorphism (SSCP) analysis, although more rapid than sequencing, is also complicated¹¹. Instead, we developed a CCR2 allele-specific assay with a rapid end-point. The principles of the spectral genotyping assay, which uses molecular beacons in combination with real-time PCR, is described more completely elsewhere¹³. Briefly, hairpin-shaped, single-stranded oligonucleotide probes are composed of a target recognition sequence and two flanking complementary sequences. A fluorophore is covalently attached to one end of the oligonucleotide, a nonfluorescent quencher to the other. Within the intramolecularly hybridized

probe, the fluorophore is close to the quencher and no fluorescence is emitted. However, when the probe hybridizes to target sequences, the quencher is removed from the proximity of the fluorophore, which then emits a detectable fluorescence signal. Genotyping of diallelic variations is achieved using two molecular beacons, each possessing a spectrally distinguishable fluorophore; for CCR2 genotyping, the wild-type allele is revealed by green fluorescence and the CCR2-64I allele by red.

The CCR2 genotype of each of 953 samples from the Chicago MACS¹⁰ was determined by spectral genotyping. To determine the specificity of the spectral genotyping assay, the genotypes of 75 random samples were also determined by DNA sequencing. Results of the two assays were in complete agreement (data not shown).

We first used the molecular beacon assay to compare the CCR2-64I genotype frequencies in HIV-1-infected and -uninfected subjects, including uninfected individuals at high risk for HIV-1 infection. There was no significant difference in CCR2-64I frequency among the different groups (Table 1), confirming previous findings that this mutant allele is not protective against HIV-1 transmission^{11,12,14}. The CCR2-64I genotype distribution is in equilibrium as predicted by the Hardy-Weinberg equation ($P > 0.5$), indicating the absence of ongoing selection for or against this allele in the study population. Overall, the CCR2 genotype frequencies we have quantitated with the spectral genotyping assay (Table 1) are very similar to those determined using direct sequencing or other methods^{11,12}.

To investigate whether the CCR2-64I allele influenced the rate of disease progression we studied the genotype frequency in seroconverter (seroincident) and seroprevalent subjects in the Chicago MACS. Because there were so few CCR2-64I homozygotes in the cohorts, we grouped together the 64I/64I homozygotes and the wt/64I

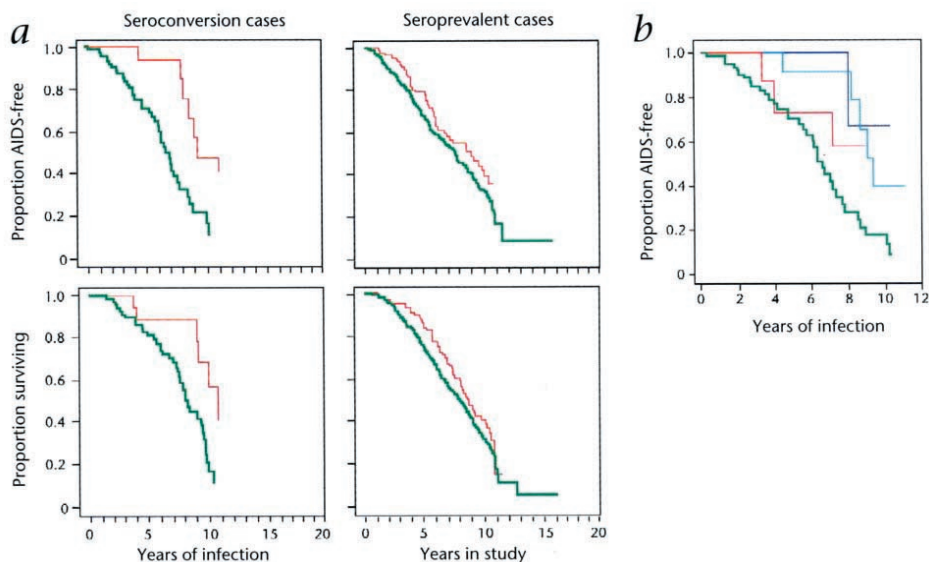
Table 1 CCR2 genotypes among HIV-1 infected and uninfected individuals

Clinical status	Number	CCR2 genotype			CCR2-64I (%) allele frequency	p value (HWE)
		+/+ (%)	+/64I (%)	64I/64I (%)		
HIV-1 infected	666	79.7	18.9	1.4	10.8	0.89
Uninfected	287	80.1	18.1	1.7	10.8	0.60
Uninfected (high risk)	35 [*]	82.9	14.3	2.9	10.0	0.47
Total	953	79.9	18.7	1.5	10.8	0.62

HWE: Hardy-Weinberg equilibrium

^{*}Uninfected (high risk) individuals are a subset of the total uninfected group who had more than 50 anal receptive intercourse events in the last two years before enrollment into the cohort.

Fig. 1 a, Effect of CCR2 genotype on disease progression. Kaplan-Meier plots for time to AIDS (upper panels) or death (lower panels) for seroconversion (left panels) and seroprevalent (right panels) individuals in the Chicago MACS. CCR2 wild-type homozygotes are indicated by green lines, CCR2-64I heterozygotes and homozygotes (grouped together) by red lines. **b**, Kaplan-Meier plots showing the effect of CCR5 and CCR2 genotypes, alone and combined, on disease progression. Green line, CCR5 and CCR2 wild-type homozygotes; red line, CCR5/ Δ 32-CCR5 heterozygotes and CCR2 wild-type homozygotes; light-blue, CCR5 wild-type homozygotes and CCR2/CCR2-64I heterozygotes; dark blue line, dual CCR5/ Δ 32-CCR5 and CCR2/CCR-64I heterozygotes.



heterozygotes, and compared them to individuals with the wt/wt CCR2 genotype. In seroconvertors, there was a clear survival benefit associated with the possession of a CCR2-64I allele (Fig. 1a, left panels), manifested by a delay of 2–3 years in the rate of progression to AIDS or death. The median survival time without AIDS in individuals with the CCR2-64I allele is 9.3 years compared to 6.7 years for wild-type individuals. The CCR2-64I allele is also a significant variable for progression to AIDS based on Cox regression analysis ($P < 0.003$); there is a reduced relative hazard of 28% for individuals with the CCR2-64I allele. When the end-point was time-to-death, the median survival time of those with one or more CCR2-64I alleles was 10.7 years, compared to 8.2 years for wild-type individuals. A Cox regression analysis ($P < 0.01$) revealed a reduced relative hazard of 32% for individuals with the CCR2-64I allele. These findings are consistent with those from other seroconvertor cohorts¹¹.

In contrast, no significant impact of the CCR2-64I allele on disease progression was observed in seroprevalent subjects (Fig. 1a, right panels) also consistent with earlier studies^{11,12,14}. The Kaplan-Meier plots are roughly parallel, and nearly overlapping, for wild-type homozygotes and those with CCR2-64I alleles. There is no statistical difference between the two groups with respect to progression to AIDS or death, although the trend is in

favor of those with the CCR2-64I allele.

The rates of CD4⁺ T-cell count decline per year (CD4 slope) were also analyzed on the basis of CCR2-64I allele distribution (Fig. 2a). Among seroconvertors, individuals with the CCR2-64I allele tended to have a distribution of CD4 slopes that was shifted towards neutral or positive values when compared with the distribution for wild-type individuals. However, the mean CD4 count declines in the two groups were not significantly different (CD4 slopes of -98 and -60 cells/ μ l/year in the wild-type and CCR2-64I groups, respectively; $P < 0.1$). In the seroprevalent cohort, the CD4 slope distribution was very similar in the two CCR2 allelic groups, and there was no statistically significant difference in the mean CD4 slopes (-74 and -50 cells/ μ l/year in wild-type and CCR2-64I individuals, respectively; $P < 0.1$).

Viral loads in plasma at the set-point 9–12 months post seroconversion were also compared with CCR2 genotype (Fig. 2b). A skew towards lower viral loads in cases with one or more CCR2-64I alleles was apparent. There was a significant difference ($P < 0.03$; Wilcoxon non-parametric rank sum test) in the viral loads of individuals with wild-type (mean = 85,000; median = 20,400 copies/ml) and CCR2-64I alleles (mean = 18,000; median = 6,500 copies/ml). This early reduction in plasma viremia in individuals with a CCR2-64I allele is

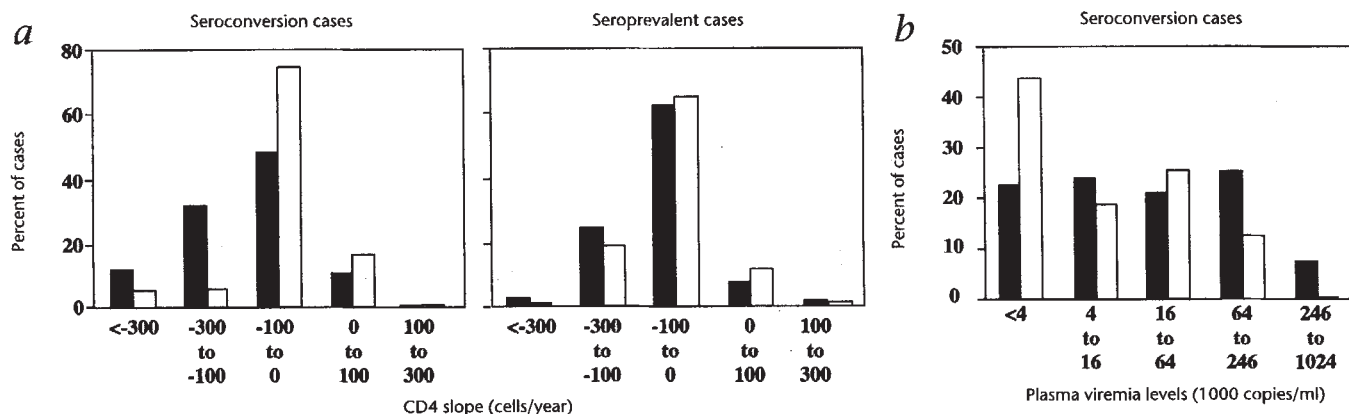


Fig. 2 a, The percentage distributions of the CD4 decline are shown for CCR2 wild-type homozygotes (■) and CCR2-64I heterozygotes and homozygotes (□).

b, The percentage distributions of plasma viremia levels 9–12 months after seroconversion in CCR2 wild-type homozygotes (black bars; $n = 74$) and in CCR2-64I heterozygotes and homozygotes (open bars; $n = 17$).

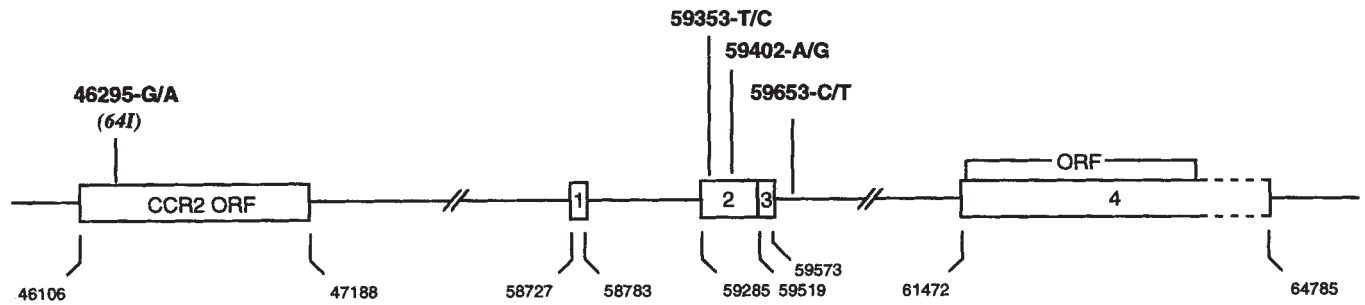


Fig. 3 Genetic association between CCR2-64I and a base substitution in the regulatory region of CCR5. A schematic representation of the genomic organization of CCR2 and CCR5 alleles is depicted. The mRNA splicing of the CCR5 allele is based on the CCR5 cDNA sequence composition previ-

ously identified¹⁵. Open boxes indicate the exons and open reading frames (ORF); lines signify introns; dotted lines signify gaps in ORF and 3'-UTR; slashed lines signify gaps in sequence. Exons are numbered based on the unpublished sequence with GenBank accession number U95626.

consistent with their reduced rate of CD4 cell loss and with their tendency not to progress as rapidly to AIDS and death.

The above observations demonstrate a protective effect of the CCR2-64I allele against disease progression, probably mediated by a reduction in plasma viremia and the rate of CD4 cell loss. How is this effect manifested biologically? It is unclear how heterozygosity for a conservative amino-acid substitution in the transmembrane region of a chemokine receptor not used for the entry of most HIV-1 strains could have a direct impact on HIV-1 replication *in vivo*. There is also no evidence that the CCR2-64I protein is dysfunctional as either a chemokine receptor or as a HIV-1 co-receptor (R. Doms, personal communication). We therefore addressed the possibility, raised by Smith *et al.*¹¹, that the CCR2-64I allele was tracking with another mutation through linkage disequilibrium.

Because of the importance of the CCR5 gene in HIV-1 entry and disease progression¹⁻³, and because CCR2 and CCR5 are in close proximity on chromosome 3, we focused on a possible association between these genes. We first determined whether the CCR2-64I genotype frequency was influenced by the presence of the $\Delta 32$ -CCR5 allele (Table 2). For the wt/wt CCR5 genotype, the CCR2 genotype frequency is in Hardy-Weinberg equilibrium. However, in CCR5/ $\Delta 32$ -CCR5 heterozygotes, there is a statistically significant reduction in CCR2-64I allele frequency ($P < 0.003$), manifested by fewer than expected wt/CCR2-64I heterozygotes and a complete absence of CCR2-64I homozygotes. That no $\Delta 32$ -CCR5 homozygotes possessed the CCR2-64I allele (Table 2), as previously observed¹¹, remains unexplained. However, similar results were obtained in studies of both HIV-1-infected and -uninfected individuals, indicating that there was no skew caused by the absence of $\Delta 32$ -CCR5 homozygotes among the HIV-1-infected cohort (Table 2)¹¹.

We next searched for a synergistic effect of CCR2-64I and $\Delta 32$ -CCR5 alleles on disease progression. A bivariate analysis of disease progression was performed by breaking down the seroconverter cohort according to the $\Delta 32$ -CCR5 and CCR2-64I genotypes.

Table 2 CCR2 and CCR5 genotype distributions in HIV-1 infected and uninfected individuals.

CCR2 genotype	CCR5 genotype					
	+/+		+/ $\Delta 32$		$\Delta 32/\Delta 32$	
+/+	620	(182)	126	(35)	10	(10)
+/64I	162	(49)	14	(2)	0	(0)
64I/64I	14	(5)	0	(0)	0	(0)

The numbers are from all individuals studied in the Chicago MACS; numbers in parentheses are from the uninfected subset.

Although the AIDS-free survival curves show that individuals who are heterozygous for the $\Delta 32$ -CCR5 allele and also have at least one CCR2-64I allele tend to progress more slowly to AIDS than individuals who are heterozygous for only one of the two mutant genes (Fig. 1b), this tendency is not statistically significant ($P < 0.09$ with Log Rank test).

In our MACS cohort, the $\Delta 32$ -CCR5 allele has a less significant effect on disease progression than the CCR2-64I allele, as is evident from the Kaplan-Meier analysis shown in Fig. 1b. Furthermore, Cox regression analysis reveals a relative risk hazard of 29% for the CCR2-64I allele, but only 46% for the $\Delta 32$ -CCR5 allele (P values of 0.01 and 0.19, respectively). Individuals who are heterozygous for both alleles ($\Delta 32$ -CCR5 and CCR2-64I) have the lowest relative risk hazard (17%). However, Cox regression analyses with various models of interaction between CCR2 and CCR5 do not show increased significance due to the interaction.

We next sequenced selected regions of the upstream non-coding regions of CCR5 (687 bp) in 124 infected and uninfected cases, taking into account the possibility of polymorphisms as described by others¹⁵. We confirmed the presence of polymorphisms in the CCR5 promoter region (59353-T/C and 59402-G/A)¹⁵, and identified a new mutation (C to T at nucleotide 59653) which was 100% associated with the G to A change that creates the CCR2-64I genotype (Fig. 3). No exceptions to this genetic association were found. We also determined the CCR5-59653C and CCR5-59653T frequencies in the MACS seroconverter cohort (117 cases) by spectral genotyping. Again, the CCR5-59653T and CCR2-64I (CCR2-46295A) genotypes corresponded 100%.

In conclusion, using spectral genotyping we confirm that the CCR2-64I allele does have a significant influence on the rate of disease progression in infected individuals but has no impact on HIV-1 transmission. The presence of the CCR2-64I allele (in both heterozygotes and homozygotes) is associated with a 2-3 year delay in progression to AIDS and deaths. This effect of CCR2-64I is only apparent in seroconvertors, and is masked in seroprevalent cases. Seroprevalent cohorts are skewed against containing rapid progressors because these individuals either died before the cohorts were formed or were not enrolled. Our observations therefore strongly suggest that seroprevalent cohorts should not be used in the future to study subtle effects of host genetics on disease progression rates by Kaplan-Meier analysis.

CCR2 and CCR5 are closely linked genes on chromosome 3, and the proteins have a high (82%) sequence homology^{11,17}. Either CCR2 or CCR5 probably arose by gene duplication. However, they now have different chemokine-binding profiles (although both re-

spond to RANTES¹⁷, and only CCR5 has been efficiently adopted by HIV-1 as a co-receptor^{2,3}. An *in vitro* correlate of the epidemiology on CCR5 is that T-cells and macrophages from $\Delta 32$ -CCR5 homozygotes resist infection by R5- (but not X4-viruses), while cells from heterozygotes have reduced susceptibility^{7,8,18,19}. However, this is not true of cells from CCR2-64I homozygotes (R. Doms, personal communication). It is not obvious how the CCR2-64I allele influences disease progression in HIV-1-infected individuals, although others have proposed plausible hypotheses¹¹. It may be that the biological effect of CCR2-64I is manifested indirectly. It is intriguing that the single nucleotide change responsible for the V64I change in CCR2 is in complete linkage disequilibrium with a polymorphism in the CCR5 regulatory region, albeit in a putative intron¹⁵. Heterozygosity for $\Delta 32$ -CCR5 reduces CCR5 surface expression by a few-fold¹⁹, but CCR5 levels on wild-type cells can vary over a 20-fold range¹⁹⁻²¹. Thus, there has been speculation about the influences of regulatory elements on CCR5 expression and the impact these could have on disease progression rates²¹. In preliminary experiments we have, however, found that the presence of a CCR2-64I allele does not significantly affect the level of CCR5 expression on CD4⁺ T-cells *in vitro* (data not shown).

The observations we have made endorse the value of screening for polymorphisms in the human genome that influence disease progression in general, and the course of HIV-1 infection in particular¹⁴. Even if the biological explanations for new polymorphisms are not immediately apparent, new lines of experimental research might emerge.

Methods

Clinical samples, virus load and CD4 slope determinations. All clinical samples were obtained from the Chicago component of the MACS and have previously been used for CCR5 genotyping¹⁰. This cohort comprises male homosexuals, approximately 90% of whom are Caucasian¹⁰. The sub-division of the 953 samples was: HIV-1 seronegative individuals ($n = 287$), HIV-1 seroprevalent individuals ($n = 549$); HIV-1 seroconvertors ($n = 117$). Plasma viremia measurements were obtained by the RT-PCR assay¹⁰.

CCR2 genotyping assay. To detect the presence of the CCR2-64I mutation (G to A substitution at position 190), we used the spectral genotyping assay. The use of molecular beacons¹⁶ in combination with real-time PCR allows the simultaneous detection of diallelic sequences which may differ only by a single nucleotide. A full description of the use of this technique to genotype CCR5 coding alleles is described elsewhere¹³. In the CCR2-64I spectral genotyping assay, we monitored the simultaneous PCR amplification of the CCR2-wild type and -64I alleles using two molecular beacons. One, labeled with FAM, was specific for the wild-type CCR2 sequence; the other, labeled with HEX, for the CCR2-64I allele. Their nucleotide sequences are, respectively: FAM-5'-CGCCGATGAGGACGACCAAGCATGGCGG-3'-DABCYL and HEX-5'-CCGCCGATGAGGATGACCAAGCATGGCGG-3'-DABCYL. The length of the target recognition sequence in the molecular beacons was designed so that they hybridize at the same temperature used for the corresponding PCR primer hybridizations. The PCR primers were 5'-GCTCTACTCGCTGTGTTCA-3' and 5'-CTCATTTGCAGCAGAGTGAGC-3'. Each 50 μ l reaction contained 1 μ g of genomic DNA, 0.25 μ M of each molecular beacon, 0.5 μ M of each primer, 0.25 μ M of each dATP, dCTP, dGTP and dTTP, 2.5 u of AmpliTaq Gold DNA polymerase (Perkin-Elmer), 50 mM KCl, 3.5 mM MgCl₂ and 10 mM Tris-HCl, pH 8.3. Sixty cycles of amplification (94 °C for 30 s, 60 °C for 60 s, and 72 °C for 3 s) were performed in a spectrofluorometric thermocycler (ABI PRISM 7700, Applied Biosystems). During the annealing stage of each cycle the fluorescence emission spectrum from 500 nm to 650 nm was recorded. After the completion of the PCR amplification, the changes in the emission spectra during the course of the amplification were analyzed. The emission spectra for each PCR cycle were decomposed into the spectral contributions of FAM and HEX. Wild-type CCR2 homozygote samples led to an increase only in the FAM component; CCR2-64I homozygotes to an in-

crease only in the HEX component; heterozygotes in both FAM and HEX. The no-template controls result in no change in fluorescence. For each sample, the threshold cycle (the cycle at which fluorescence exceeds a defined value over the background fluorescence) of FAM is plotted against the threshold cycle of HEX.

Genotyping of CCR5 regulatory region. The CCR5-59653 mutation is a C to T substitution at position 59653 of the unpublished sequence of the 5'-untranslated region of CCR5 (GenBank accession number, U95626). Blood samples were obtained from 33 HIV-1-infected and 91 uninfected individuals. Genomic sequences of 688 nucleotides long spanning the CCR5 5'-untranslated region and the two noncoding exons¹⁵ were amplified from each sample by PCR. The primers were LK84 (5'-AAGTCCAGGATCCCCCTCTA-3' at positions 59043-59064) and LK87 (5'-CATTCCAACTGTGACCCCTTCC-3' at positions 5909-59732). PCR was performed as described above, using 40 cycles of (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s). Each PCR-amplified DNA was purified and sequenced using LK84, LK85 (5'-GTGTAGTGGGATGACGAGAGA-3' at positions 59290-59550) and LK86 (5'-CAGAAGAGCTGAGACATCCGT-3' at positions 59530-59550) as overlapping sequencing primers. Sequence reactions were performed according to the dideoxy-chain-termination method using the automated 377 DNA Sequencer (Applied Biosystems).

Acknowledgements

We thank Sunil Ahuja for communication of unpublished results and helpful discussions, and Rhonda Kost for clinical assistance. Supported by the National Institutes of Health under awards AI45218, AI40387, AI35039, AI42454 and AI41420, and by the Irene Diamond Fund. J.P.M. is an Elizabeth Glaser Scientist and L.G.K. is an Aaron Diamond Fellow.

RECEIVED 26 NOVEMBER 1997; ACCEPT 3 FEBRUARY 1998

- Fauci, A.S. Host factors and the pathogenesis of HIV-induced disease. *Nature* **384**, 529-534 (1996).
- Moore, J.P., Trkola, A. & Dragic, T. Co-receptors for HIV-1 entry. *Curr. Opin. Immunol.* **9**, 551-562 (1997).
- Berger, E.A. HIV entry and tropism: the chemokine receptor connection. *AIDS* **11** (suppl A), S3-S16 (1997).
- Berger, E.A. et al. HIV-1 phenotype classified by co-receptor usage. *Nature* **391**, 240 (1998).
- Zhu, T. et al. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* **261**, 1179-1181 (1993).
- Richman, D.D. & Bozzette, S.A. The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression. *J. Inf. Dis.* **169**, 968-974 (1994).
- Liu, R. et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367-378 (1996).
- Samsom, M. et al. Resistance to HIV-1 infection of Caucasian individuals bearing mutant alleles of the CCR5 chemokine receptor gene. *Nature* **382**, 722-725 (1996).
- Dean, M. et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion of the CCR5 structural allele. *Science* **273**, 1856-1862 (1996).
- Huang, Y. et al. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nature Med.* **2**, 1240-1243 (1996).
- Smith, M.W. et al. Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. *Science* **277**, 959-965 (1997).
- Michael, N.L. et al. The role of CCR5 and CCR2 polymorphisms in HIV-1 transmission and disease progression. *Nature Med.* **3**, 1160-1162 (1997).
- Kostrikis, L.G., Tyagi, S., Mhlanga, M.M., Ho, D.D. & Kramer, F.R. Spectral genotyping of human alleles. *Science* (in the press).
- Smith, M.W. et al. CCR2 chemokine receptor and AIDS progression. *Nature Med.* **3**, 1052-1053 (1997).
- Mummidi, S., Ahuja, S.S., McDaniel, B. & Ahuja, S.K. The human CC chemokine receptor 5 (CCR5) gene: Multiple transcripts with 5'-end heterogeneity, dual promoter usage, and evidence for polymorphisms within the regulatory regions and noncoding exons. *J. Biol. Chem.* **272**, 30662-30671 (1997).
- Tyagi, S. & Kramer, F. Molecular beacons: probe that fluorescence upon hybridization. *Nature Biotech.* **14**, 471-476 (1996).
- Premack, B.A. & Schall, T.J. Chemokine receptors: gateways to inflammation and infection. *Nature Med.* **2**, 1174-1178 (1996).
- Paxton, W.A. et al. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposures. *Nature Med.* **2**, 412-417 (1996).
- Wu, L. et al. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, *in vitro*. *J. Exp. Med.* **185**, 1681-1691 (1997).
- Trkola, A. et al. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR5. *Nature* **384**, 184-186 (1996).
- Moore, J.P. Co-receptors: Implications for HIV pathogenesis and therapy. *Science* **276**, 51-52 (1997).