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Role of CCR2 Genotype in the Clinical Course of Syncytium-Inducing (SI) or Non-SI Human Immunodeficiency Virus Type 1 Infection and in the Time to Conversion to SI Virus Variants

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The effect of a valine to isoleucine switch in the CCR2 first transmembrane domain (CCR2 64I) on the clinical course of human immunodeficiency virus type 1 (HIV-1) infection was analyzed in relation to the presence or absence of syncytium-inducing (SI) HIV-1 variants. Compared with persons with a wild-type genotype for CCR2 and CCR5, subjects with a CCR2-64I/1 or 64I/64I (but CCR5 wild-type homozygous genotype) had significantly delayed disease progression (relative hazard, 0.66; 95% confidence interval, 0.44–0.99) with a 1.5-fold slower CD4 T lymphocyte decline and a 1.2-fold lower RNA virus load. The delay in disease progression was more pronounced when only non-SI (NSI) HIV-1 variants were present and was not observed after conversion to SI HIV-1 in CCR2-64I/1 persons. In CCR2-64I/1 subjects, a higher conversion rate to and a higher prevalence of SI HIV-1 was observed. These findings suggest that the mechanism of action of the CCR2 polymorphism is mediated via CCR5-restricted NSI HIV-1 variants.

G protein-coupled seven-transmembrane receptors have been identified as the coreceptors for human immunodeficiency virus type 1 (HIV-1). Syncytium-inducing (SI), T cell line-adapted HIV-1 variants utilize the C-X-C chemokine receptor 4 (CXCR4), whereas macrophage-tropic, non-SI (NSI) HIV-1 variants use the C-C chemokine receptor 5 (CCR5), and some primary SI viruses may use both. Other receptors, including CCR2 and CCR3, also serve as coreceptors, albeit for a minority of HIV-1 variants [1, 2].

A specific 32-bp deletion in the CCR5 gene (CCR5 Δ32) has been associated with a reduced susceptibility for infection and delayed disease progression [3]. This has been confirmed in many cohorts, including the Amsterdam Cohort [4]. More recently, a valine to isoleucine switch in the first transmembrane domain of CCR2 (CCR2 64I) was shown to be associated with delayed disease progression [5–7].

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Written informed consent was obtained from all participants. In the conduct of clinical research, human experiment guidelines of the authors’ institutions were followed.

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Here, we analyze the role of CCR2 genotype in the clinical course of HIV-1 infection in Amsterdam Cohort Studies participants in relation to the presence or absence of SI HIV-1 variants. We also analyzed the conversion rate towards SI HIV-1 variants in relation to CCR2 genotype.

Methods

Study population. The study population, 364 Caucasian, homosexual men enrolled in the Amsterdam Cohort between October 1984 and March 1986, was previously described [4]. The censor date of our study was set at 1 January 1996. None of the participants received triple therapy during follow-up. Of the 364 participants, 131 seroconverted during the study. No differences in AIDS-free survival were found between persons who had documented seroconversion and seroprevalent persons by Kaplan-Meier (P = .36) and Cox proportional hazard analysis (relative hazard [RH], 1.17; 95% confidence interval [CI], 0.84–1.63) for seroconverter compared with seroprevalent persons. This result suggests a good estimation of the seroconversion date in the latter group. Therefore, we combined 131 persons with documented seroconversion and 233 seroprevalent subjects as one study group.

CCR2 genotyping. DNA samples were available for CCR2 genotyping from 344 (94%) of the 364 men. Genomic DNA was
lymphocyte counts had to be available for analysis; 320 of 364 lymphocytes was determined separately by fitting a simple regression. PCR was done in a thermocycler (Uno II; Biometra, Göttingen, Germany) as follows: 5 min of denaturation at 95°C; 35 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C (slope 2°C/s), and 5 min of elongation at 72°C. PCR products were subjected to restriction analysis with BsmBI for 2 h at 60°C (New England BioLabs, Beverly, MA) and analyzed on a 4% Nusieve gel (2:2). Fifteen samples (11 CCR2 wild-type and 4 CCR2 64I heterozygous samples) were subjected to sequence analysis (Thermo sequenase-radiolabeled terminator cycle sequence kit; Amersham, Cleveland), which confirmed the CCR2 genotype as determined by PCR-RFLP analysis (not shown).

Virolologic assays. HIV-1–positive peripheral blood mononuclear cells were cocultivated with MT2 cells every 3 months to detect SI HIV-1 variants [9]. Time of SI conversion was the calculated midpoint between last SI-negative and first SI-positive sample. SI phenotype data were available for 281 of 364 participants. Serum virus load was measured by use of a quantitative HIV-1 RNA nucleic acid–based sequence amplification (NASBA QT; Organon Teknika, Boxtel, The Netherlands). Vireal RNA load levels were measured as a product of 2.3 years (range, 1.5–3.0) after seroconversion. Serum samples were available for 335 (92%) of 364 participants. The number of RNA copies below the test threshold of quantification was arbitrarily set at 10^3/mL.

Statistical analysis. We used the χ^2 test to compare HIV-1–positive study populations for CCR2 64I allele frequency. The Mann-Whitney U test was used to compare CCR2 and CCR5 genotypic groups. Kaplan-Meier and Cox proportional hazard analyses were done to study the effect of CCR2 and CCR5 genotype in HIV-1 disease progression. Significance in survival analyses was determined by log-rank test.

For each participant, the slope of the decrease in CD4^+ T lymphocytes was determined separately by fitting a simple regression line to his CD4^+ T lymphocyte counts. At least 3 CD4^+ T lymphocyte counts had to be available for analysis; 320 of 364 participants had sufficient counts.

Kaplan-Meier analysis was used to estimate the cumulative incidence of conversion to SI HIV-1 variants. In the analysis considering the period in which only NSI HIV-1 variants are present, conversion to SI HIV-1 was used as a censor criterion. We used a software program for statistical analysis (version 6.0; Number Crunching Statistical Systems, Klaysville, UT).

Results

CCR2 genotype distribution. In the same study population, we previously determined the CCR5 Δ32 genotype distribution [4]. These data were combined here with the CCR2 genotype data. Of the HIV-1–seropositive participants, 45 (13.1%) were heterozygous (CCR2-64I+/+) and 5 (1.5%) were homozygous (CCR2-64I/64I) for the CCR2 64I allele and homozygous for the common CCR5 allele (CCR5-+/+). Sixty-two subjects (18.0%) were heterozygous for CCR5 Δ32 and homozygous for the common CCR2 allele (CCR2-+/+, CCR5-Δ32/Δ32); 6 subjects (1.7%) were heterozygous for both CCR2 64I and CCR5 Δ32 (CCR2-64I/+, CCR5-Δ32/Δ32). The remaining 226 subjects (65.7%) were homozygous for both the common CCR2 and CCR5 allele (CCR2-++/+, CCR5-+++/+). The CCR2 allelic frequency was 0.089, which was not significantly different from 0.098, which was reported by Smith et al. [5] for another Caucasian HIV-1–seropositive population (P = .48).

As shown by Smith et al. [5], the mutant alleles of CCR2 and CCR5 are in strong linkage disequilibrium: CCR2-64I only occurs on a CCR5– Δ32 chromosomal haplotype and CCR5-Δ32 only occurs on a CCR2– Δ32 chromosomal haplotype. This means that the polymorphisms in CCR2 and CCR5 can be seen as three alleles of a combined CCR2/CCR5 locus, in which [+I+] represents CCR2-+/+ and CCR5-Δ32/Δ32, [64I/+] represents CCR2-64I/Δ32/Δ32 and CCR5-Δ32/Δ32, [64I/64I] represents CCR2-64I/64I and CCR5-Δ32/Δ32, [Δ32/+] represents CCR2-++/Δ32 and CCR5-Δ32/Δ32, and [Δ32/64I] represents CCR2-64I/Δ32 and CCR5-Δ32/64I genotypes. The CCR2/CCR5 compound genotypes are used throughout our analyses.

CCR2 and CCR5 genotype and HIV-1 clinical course. Univariate Cox proportional hazard analysis showed an RH (95% CI) for progression to AIDS (CDC 1987 definition [10]) of 0.67 (0.44–1.01), 0.59 (0.16–2.14), 0.39 (0.26–0.58), and 0.16 (0.04–0.63) for genotypes CCR2/CCR5-[64I/+] [64I/64I], [Δ32/Δ32], and [Δ32/64I], respectively, compared with genotype CCR2/CCR5-Δ32/Δ32. The similar RHs for CCR2 64I homo- and heterozygosity prompted us to combine these subjects into 1 genotypic group in further analyses. This combined group showed an RH for progression to AIDS of 0.66 (95% CI, 0.44–0.99). Because few persons were heterozygous for both CCR2 64I and CCR5 Δ32, these subjects were excluded from the Kaplan-Meier analyses.

Kaplan-Meier analysis of all 344 HIV-1–seropositive participants with a known CCR2 and CCR5 genotype showed a significantly prolonged AIDS-free survival (1987 definition) both for CCR2/CCR5-[64I/+] (P = .04) and for CCR2/CCR5-[Δ32/+] subjects (P < .001) compared with CCR2/CCR5-Δ32/Δ32 subjects (figure 1, top). Similar results were obtained when we used the 1993 AIDS definition [11] as an end point ([64I/+] RH = .06; RH = 0.70 [95% CI, 0.48–1.02]; [Δ32]: P < .001, RH = 0.41 [95% CI, 0.28–0.61]).

When death was used as an end point in Kaplan-Meier analysis, a significantly delayed disease progression was observed both for CCR2/CCR5-[64I/+] (P = .03; RH = 0.62 [95% CI, 0.36–0.97]) and [Δ32/+] subjects (P < .001; RH = 0.42 [95% CI, 0.27–0.64]) compared with CCR2/CCR5-Δ32/Δ32 subjects (figure 1, bottom). No differences were observed in survival after AIDS diagnosis in CCR2/CCR5-64I+/+ subjects (data not shown).

In accordance with a delayed disease progression, a signifi-
cantly slower decrease in CD4 T lymphocytes was seen in CCR2/CCR5-[64I/+] (decrease of 53/mm³/year \( P = .005 \)), [Δ32/+] (53/mm³/year \( P = .004 \)), and [64I/Δ32] subjects (27/mm³/year \( P = .02 \)) compared with CCR2/CCR5-[+/+] subjects (78/mm³/year). The median virus load 2 years after seroconversion in CCR2/CCR5-[+/+] subjects was 10^{4.4} copies/mL (range, 10^{3.0}–10^{6.0}); this level was 1.3-fold higher (\( P = .41 \)) than the viral RNA load in [64I/+] subjects (10^{4.3} copies/mL [range, 10^{3.9}–10^{6.1}]), 2.5-fold higher (\( P = .02 \)) than in [Δ32/+] subjects (10^{6.0} copies/mL [range, 10^{5.0}–10^{6.0}]), and 20.0-fold higher (\( P = .008 \)) than in the small group of CCR2/CCR5-[64I/Δ32] subjects (10^{3.1} copies/mL [range, 10^{3.0}–10^{4.1}]).

**CCR2 and CCR5 genotype in relation to HIV-1 biologic phenotype.** At the end of the study, not considering subjects lost to follow-up, the total frequency of SI HIV-1 was higher in CCR2/CCR5-[64I/+] subjects (21 [52.5%] of 40) than in subjects categorized as [Δ32/+] (20 [37%] of 54), [64I/Δ32] (1 [20%] of 5), and CCR2/CCR5-[+/+] (61 [33.9%] of 180). The prevalence of SI HIV-1 in CCR2/CCR5-[64I/+] and [+/+] subjects was significantly higher than the prevalence in [+/+] persons (\( P = .03 \)).

To analyze a possible difference in the rate of evolution to SI HIV-1, Kaplan-Meier survival analysis was performed with conversion to SI HIV-1 as an end point (figure 2, top). In the

**Figure 1.** CCR2 and CCR5 genotype and disease progression. Kaplan-Meier plots for time to AIDS (1987 definition; top) or time to death (bottom) for subjects classified as CCR2/CCR5-[64I/+] (thick lines), [Δ32/+] (thin lines), and [+/+] (intermediate lines). No. of men at risk are indicated for each time point. Relative hazards (RH), confidence intervals (CI), and log-rank \( P \) values are shown.
Figure 2. CCR2 and CCR5 genotype and HIV-1 biologic phenotype. Kaplan-Meier plots for subjects classified as CCR2/CCR5$^{[+/-]}$ (intermediate lines), $^{[64I/+]}$ (thick lines), and $^{[D32/+]}$ (thin lines) for time to emergence of SI HIV-1 variants (top), time to AIDS when only NSI variants were present (middle), and time to AIDS after emergence of SI HIV-1 (bottom). No. of men at risk are indicated for each time point. Relative hazards (RH), confidence intervals (CI), and log-rank $P$ values are shown.
first 4.5 years after seroconversion, ~15% of subjects in each of the 3 genotypic groups converted to an SI HIV-1 variant. During the subsequent follow-up, however, CCR2/CCR5-[64I/+] subjects had an increased conversion rate. Median time to SI conversion was 8.5 years after seroconversion in CCR2/CCR5-[64I/+] subjects but 11.5 years in CCR2/CCR5-[Δ32I/+] and [+/+] subjects. However, the difference in evolution to an SI phenotype between CCR2/CCR5-[64I/+] and [+/+] subjects was not significant for the total follow-up period (P = .23). The previously described more rapid evolution to SI HIV-1 variants in the group with CCR5-[Δ32]-homozygotes compared with CCR5-[Δ32] heterozygotes [4] was still visible, although not statistically significant (P = .31).

The protective effects of CCR2 64I and CCR5 Δ32 were stronger only in the presence of NSI HIV-1 variants when compared with total follow-up (figure 2, middle; [64I/+]: RH = 0.44 [95% CI, 0.22–0.90] vs. RH = 0.66 [95% CI, 0.44–0.99]; [Δ32I/+] RH = 0.22 [95% CI, 0.11–0.42] vs. 0.40 [95% CI, 0.26–0.58]). Analysis of the period after the emergence of SI HIV-1 variants (figure 2, bottom) showed that CCR5-Δ32 heterozygosity was still associated with prolonged AIDS-free survival (P = .06; RH = 0.44 [95% CI, 0.20–0.97]). The protective effect of CCR2 64I remained evident but was no longer significant after the emergence of SI (P = .44; RH = 0.73, [95% CI, 0.34–1.54]).

Discussion

We confirmed a correlation between CCR2 genotype and prolonged AIDS-free survival, as originally described by Smith et al. [5]. In 344 HIV-1-seropositive participants from the Amsterdam Cohort Studies, a Cox proportional hazard analysis on CCR2/CCR5-[64I/+] genotype yielded an RH of 0.66 for progression to AIDS. This confirms the findings in various cohorts in which RH varied from 0.6 to 0.7 for the CCR2 64I heterozygous genotype [5, 6]. In accordance with a delayed progression to AIDS, we observed a slower CD4 cell decline and a lower serum viral RNA load in CCR2/CCR5-[64I/+] than in [+/+] subjects.

The biologic mechanism by which the CCR2 64I genotype provides protection against disease progression is not known. Primary HIV-1 variants in general do not use CCR2 ([12, 13], A.M.R.H., unpublished data) excluding coreceptor dysfunctioning as a major explanation. More likely, this polymorphism influences the CCR5 pathway, directly or indirectly. It has been suggested that CCR2 64I may result in a diminished expression of CCR5. In support of this is the observation that the G to A mutation resulting in the CCR2 64I substitution is linked to a point mutation in the promoter region of the CCR5 gene [6]. However, this point mutation is located within a 1.9-kb intron that may be dispensable for optimal promoter activity [14, 15]. In agreement with this, neither preliminary data from Kostrikis et al. [6] or Rizzardi et al. [7] nor preliminary results from our laboratory (A.M.R.H.) support the hypothesis that CCR5 expression is reduced in CCR2 64I heterozygotes.

In this study, we showed a higher conversion rate to and a higher prevalence of SI HIV-1 variants among CCR2 heterozygotes than in wild-type homozygotes in contrast to CCR5 Δ32 heterozygotes in which SI conversion tends to be delayed. This suggests a different mechanism of action of CCR2 64I than of CCR5 Δ32 in delaying HIV-1 disease progression.

The protective effects of both CCR2 64I and CCR5 Δ32 were stronger in the sole presence of NSI HIV-1 variants when compared with the total follow-up. This suggests that the mechanism of both protective polymorphisms is mediated via NSI HIV-1 variants, which in general are CCR5 restricted. Elucidating the mechanism of action of CCR2 64I might help to identify potential sites for therapeutic interventions in HIV-1 infection.

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