Combined Effect of CCR5-Δ32 Heterozygosity and the CCR5 Promoter Polymorphism −2459 A/G on CCR5 Expression and Resistance to Human Immunodeficiency Virus Type 1 Transmission

Florian Hladik,[1]† Huanliang Liu,[2]†‡ Emily Speelmon,[1] Devon Livingston-Rosanoff,[1]
Sean Wilson,[1] Polachai Sakchalathorn,[1] Yon Hwangbo,[2] Benjamin Greene,[2]
Tuofu Zhu,[2,3] and M. Juliana McElrath[1,2,4]*

*Program in Infectious Diseases, Clinical Research Division, Fred Hutchinson Cancer Research Center,[1] and
Departments of Laboratory Medicine,[2] Microbiology,[3] and Medicine,[4] University of Washington
School of Medicine, Seattle, Washington*

Exposed seronegative individuals (ES) with persistent high-risk sexual behavior may be less susceptible to human immunodeficiency virus type 1 (HIV-1) infection because they carry the chemokine receptor (CR) gene alleles CCR5 open reading frame (ORF) Δ32, CCR5 promoter −2459G, or CCR2 ORF 64I (CCR2-64I), all of which have been found to diminish HIV-1 infectivity and/or disease progression. To investigate this, we determined the haplotypes for these three genetic loci in 93 ES and 247 low-risk control individuals. To test if protective haplotypes exert their effect by modulating CR expression, we measured the protein expression of CCR5 and CXCR4 on circulating CD4+ T cells and CD14+ monocytes in 71 ES and 92 controls. To avoid investigator bias, the analysis was performed without knowledge of each subject's risk and genotype. The CCR5 −2459G allele was significantly enriched in ES Caucasian men, who constituted the majority (84%) of the ES cohort, compared to the control Caucasian men ($P = 0.02$). This increase was mostly attributable to a higher frequency of the −2459 A/G versus the −2459 A/A genotype in individuals heterozygous for the Δ32 allele ($P = 0.012$). No protective influence of the CCR2-64I allele was observed. The haplotypes CCR5 ORF Δ32/CCR5 −2459A (in complete linkage disequilibrium) and CCR5 ORF wt/CCR5 −2459G had a cumulative negative effect on the expression of CCR5, since we measured significantly reduced CCR5 densities on both T-helper cells and monocytes only when both haplotypes were present. Densities of CCR5 on lymphocytes and monocytes were correlated ($r = 0.59$; $P < 0.0001$), indicating concordance of CCR5 expression patterns across different cell types. We conclude that the CCR5 ORF Δ32/wt-CCR5 −2459 A/G genotype combination offers an advantage in resisting sexual HIV-1 transmission and that this effect is mediated by a relative paucity of CCR5 on potential target cells of HIV-1.

Human immunodeficiency virus type 1 (HIV-1) requires expression of CD4 in conjunction with a chemokine coreceptor, most frequently CCR5 or CXCR4, to productively infect target cells. Since the discovery of these coreceptors, it has become well established that alterations in their gene expression and function can impact HIV-1 disease progression. For example, inheritance of the chemokine receptor (CR) polymorphisms CCR5 open reading frame (ORF) Δ32, CCR5 promoter −2459 A-to-G, and CCR2 ORF 190 G-to-A (CCR2-64I) are associated with delayed development of the AIDS in HIV-1-infected patients (4, 5, 9, 14, 16, 17, 20, 21, 29, 33, 35, 36, 38, 39, 45, 53, 54). Moreover, individuals homozygous for the CCR5-Δ32 allele, comprising about 1% of the Caucasian population, have strongly reduced susceptibility to R5-dependent HIV-1 infection (7, 26, 49). However, it is clear that the infrequent inheritance of homozygosity for the CCR5-Δ32 allele cannot account for the majority of persons worldwide who are repeatedly exposed to HIV-1 by high-risk activities but remain seronegative and uninfected.

In this study, we investigated the contributions of the CCR5 −2459G and CCR2-64I polymorphisms, as well as heterozygosity for CCR5-Δ32, to protection against HIV-1 infection in a well-defined exposed seronegative (ES) longitudinal cohort (8, 12) in contrast to a low-risk uninfected control group. Whereas CCR5 ORF Δ32/Δ32 homozygosity occurs in just 1% of Caucasians, the CCR5 −2459 A/G plus CCR5 ORF Δ32/wt or CCR2 ORF 64I/wt genotype combinations comprise approximately 5% each. However, the effects of these two genotype combinations on the susceptibility to HIV-1 infection in vivo have not been conclusively determined (6, 7, 13, 14, 17, 22, 27, 30, 31, 37, 49, 53, 55, 61). Here, we have compared the frequencies of the CCR5 ORF wt/Δ32, CCR5 −2459A/G, and CCR2 ORF 64V/I alleles and allele combinations between the ES and the low-risk control group. In addition, we determined the impact of inheriting specific haplotypic combinations of these alleles in our cohorts on the expression of CCR5 on HIV-1 target cells. Our results indicate that inheritance of the

* Corresponding author. Mailing address: Fred Hutchinson Cancer Research Center, Program in Infectious Diseases, 1100 Fairview Ave. N., P.O. Box 19024, D3-100, Seattle, WA 98109-1024. Phone: (206) 667-6704. Fax: (206) 667-4411. E-mail: kd@u.washington.edu.
† F.H. and H.L. contributed equally to this work.
‡ Present address: Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL 33136.

CCR5 ORF Δ32/wt plus −2459 A/G genotype combination occurs more commonly in ES than in low-risk control individuals and that these genotypes together may act to reduce target cell susceptibility to HIV-1.

MATERIALS AND METHODS

Study populations. Ninety-three healthy HIV-1-seronegative individuals ≥18 years old who reported high-risk sexual activity with known HIV-1-infected partners were recruited within metropolitan Seattle for longitudinal study at the Fred Hutchinson Cancer Research Center HIV-1 Vaccine Trials Unit. Enrollment criteria were unprotected sexual intercourse with a known HIV-1-infected person ≥6 times in the previous 6 months or an average of twice weekly ≥4 months within 2 years of enrollment. Prior receipt of an HIV-1 vaccine was an exclusion criterion. Volunteers entering the study were designated ES. A medical history, physical examination, complete blood cell count, T-cell subset analysis, and HIV-1 testing were done at the screening visit to confirm eligibility. HIV-1 infection was excluded by the following tests done at the screening visit and study day 0: HIV-1/2 enzyme-linked immunosorbent assay (ELISA) and Western blot (50), HIV-1 plasma RNA reverse transcription-PCR (Amplicor HIV-1 Monitor; Roche Molecular Systems) (19), and peripheral blood mononuclear cell (PBMC) DNA PCR (41). HIV-1/2 ELISA and Western blot were repeated every 3 months, and HIV-1 PBMC DNA PCR was performed again 4 weeks and 8 weeks after enrollment and then repeated yearly. Clinicians performed HIV-1 risk reduction counseling during each visit. The volunteers completed a questionnaire concerning risk behavior and provided blood during visits scheduled every month over the initial 3 months and every 3 to 6 months thereafter.

A control group of 247 HIV-1-seronegative individuals ≥18 years old reporting HIV low-risk sexual activities were also recruited. Each ES and control volunteer provided written consent prior to enrollment. All aspects of the study were approved by the Institutional Review Board at the University of Washington and the Fred Hutchinson Cancer Research Center.

Genotyping of genetic polymorphisms. PBMC were isolated by Ficoll-Hypaque density centrifugation, and PCR and DNA restriction fragment length polymorphism analyses were performed as previously described for genotyping of the CCR5 ORF Δ32 (43), CCR5 promoter −2459G (23), and CCR2 ORF 64I (37) polymorphisms (25). The forward primer for CCR2 ORF 64I was slightly modified from the original publication to 5′-TTTTGTGGGCAACATGATG G-3′ to achieve clearer separation of the bands on the gel that are characteristic of the wild type (wt) or the 64I allele.

Flow cytometric analysis of CCR5 and CXCR4. In preliminary experiments, the saturating concentrations of the anti-CCR5 and anti-CXCR4 monoclonal antibodies were determined (2.5 μg/ml) and used throughout the study. PBMC were incubated with either anti-CCR5 (clone 2D7), anti-CXCR4 (clone 12G5), or immunoglobulin G2a (IgG2a) isotype control (all BD Pharmingen) in phosphate-buffered saline supplemented with 1% bovine serum albumin and 0.1% sodium azide (fluorescence-activated cell sorting [FACS] buffer) for 30 min at 4°C. After two washes in FACS buffer, cells were incubated in fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG Fc F(ab′)$_2$ (1:100; Qifi-kit; DAKO) for 20 min at 4°C. Cells were washed twice, and a final incubation step was performed with a combination of either phycoerythrin (PE)-conjugated anti-CD3 and allophycocyanin (APC)-conjugated anti-CD4 or anti-CD14-PE and anti-CD8-APC (all BD Pharmingen). 7-Aminoactinomycin D (7-AAD; Sigma) (1 μg/ml) was added during this step to mark dead cells. Two final washes were performed in FACS buffer containing 10 μg/ml actinomycin D (Sigma) to prevent leakage of 7-AAD from dead cells. Cells were fixed in 2% paraformaldehyde containing 5 μg/ml actinomycin D and analyzed on a Calibur flow cytometer (Becton Dickinson) within 5 days after staining. 7-AAD$^+$ dead cells were excluded from the analysis, and the percentages of CCR5- and CXCR4-expressing CD3$^+$ CD4$^+$-T-helper lymphocytes and CD14$^+$ monocytes were determined.

The receptor densities of CCR5 and CXCR4 per cell were quantified by comparing FITC staining of cells to a standard curve, established by analysis of a mixture of five calibrated bead populations coated with five different quantities of immunoglobulins/bead (Qifikit). Beads were incubated with the FITC-conjugated goat anti-mouse IgG Fc F(ab′)$_2$ in parallel to the samples and acquired with identical fluorescence gain and compensation settings. The fluorescence intensity of each bead population was plotted against its designated number of immunoglobulins/bead, and the five data points were interpolated by linear regression. CD3$^+$ CD4$^+$-T-lymphocyte and CD14$^+$ monocyte populations were defined as CCR5$^+$ or CXCR4$^+$ by comparison to the corresponding isotype control, and the regression formula was used to convert the geometric mean of the fluorescence intensity of these CCR5$^+$ or CXCR4$^+$ cell populations to the mean density of receptors per cell.

In vitro HIV-1 infection of CD4$^+$ T lymphoblasts. The R5-tropic molecular clone HIV-1$_{JR-CSF}$ was used for infectivity studies, grown from the proviral plasmid pYK-JR-CSF (3) (NIH AIDS Research and Reference Reagent Program) by calcium phosphate-mediated transfection of HEK293T cells. Two days after transfection, the cell-free virus supernatant was collected, and the virus titer was determined on multinuclear activation of galactosidase indicator (MAGI) cells (58) transduced with CCR5, which were kindly provided by Michael Emerman (Fred Hutchinson Cancer Research Center, Seattle, Wash.).

Cryopreserved PBMC from ES and control individuals were allowed to rest overnight and then were depleted twice of CD8$^+$ T lymphocytes by incubation with anti-CD8 antibody-coated beads and magnetic column depletion (Miltenyi). CD4-enriched cells were stimulated for 3 days with 1.5 μg/ml phytohemagglutinin (PHA; Remel) in HEPES-buffered RPMI 1640 supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine (all Gibco), and 10% heat-inactivated fetal bovine serum (Gemini Bio-Products) (R-10). Following stimulation, nonadherent cells were removed, washed twice, and resuspended in R-10 containing 100 U/ml interleukin-2 (Chiron) (R10-100). PHA-stimulated CD4$^+$-enriched lymphoblasts were incubated with HIV-1$_{JR-CSF}$ at multiplicities of infection (MOI) of 0.003 and 0.006 for 4 h at 37°C. Cells were washed three times and plated in quadruplicates for each MOI into a 96-well round-bottom tissue culture plate at 2×10^5 cells and 200 μl R10-100 per well. On days 3 and 5, 100 μl of medium was replaced. Seven days after infection, supernatants were harvested for HIV-1 p24 determination by ELISA (Perkin-Elmer).

Statistical analysis and specimen blinding. Proportions of individuals harboring a specific genotype or genotype combination were compared between ES and control individuals by the chi-square test.

Flow cytometric analysis was done without knowledge of the risk assignment of the samples, and data were unblinded only upon completion of the study. In some samples, very low percentages of positive cells were observed, which indicated low receptor expression levels. However, the scarcity of positive cells in these cases did not permit an accurate determination of the receptor density. This problem occurred when the percentage of positive cells was <1%; for the statistical analysis, these samples were assigned a density equal to the mean density of all other values in the group − 2 standard deviations. Among the 163 specimens that were evaluated by flow cytometry, this occurred once each for CCR5 and CXCR4 expression on CD4$^+$ T cells and 19 times and 23 times for CCR5 and CXCR4 expression on monocytes, respectively.

Percentages and densities of CCR5 and CXCR4 expression were compared between multiple groups harboring different genotype combinations using the Kruskal-Wallis statistic. If a significant difference was detected by the Kruskal-Wallis test (KW) ($P \leq 0.05$), then individual two groups were compared by Dunn's multiple comparison and Mann-Whitney test. If a difference was significant by the Mann-Whitney test ($P \leq 0.05$), but not by the Dunn's test ($P > 0.05$), which adjusts for multiple comparisons, it was considered a trend. Correlations between lymphocyte densities and either monocyte densities or lymphoblast infectivities were tested by Spearman correlation.

RESULTS

Relative resistance to HIV-1 infection in exposed seronegative individuals with continuing high-risk behavior. The ES study population consisted of 93 individuals with an identified HIV-1-infected sexual partner who met the enrollment criteria and had no evidence of HIV-1 infection by serology and PCR testing at the time of recruitment. Seventy-eight (84%) reported unprotected anal or vaginal intercourse six times or more during the 6 months prior to enrollment, and 11 (12%) engaged in these activities a minimum of twice weekly for ≥4 months within the past 2 years. The median age at study entry was 37.4 years (range, 20.3 to 60.2 years). Eighty-eight ES were Caucasian (95%), 3 ES were Hispanic, 1 ES was African-American, and 1 ES was American Indian/Alaska Native. Eighty-one ES were male (87%), and of these, 76 (82% of all) ES were men having sex with men (MSM); 12 ES were female (13%). Eleven men and one woman also acknowledged injection drug use either at screening or during follow-up.

TABLE 1. High-risk activities of 93 exposed
seronegative individuals

Parameter	No. of individuals
Frequency of unprotected intercourse	
With known HIV-1-infected partner since enrollment	
During first 6 mo of study ($n = 93$)	
≥6	37
1–5	24
0	32
During last 6 mo of study ($n = 75$)[a]	
≥6	21
1–5	14
0	40
With partner of unknown HIV-1 status since enrollment	
During first 6 mo of study ($n = 93$)	
≥6	22
1–5	12
0	59
During last 6 mo of study ($n = 75$)[a]	
≥6	13
1–5	12
0	50
No. of sexual partners	
During first 6 mo of study ($n = 93$)	
≥5	44
2–4	12
1	32
0	5
During last 6 mo of study ($n = 75$)[a]	
≥5	35
2–4	7
1	33
0	0

[a] Excludes individuals who were observed for <1 year.

The ES individuals were followed for a median of 102.6 weeks. The majority (66%) reported unprotected sexual intercourse with known HIV-1-infected partners during the first 6 months of the study, and 47% followed for ≥1 year continued this practice (Table 1). In addition, approximately one-third reported high-risk sexual activity with partners of unknown HIV-1 status over the course of the study (Table 1). The total number of sexual partners also remained stable over the study period (Table 1). Additional details of high-risk sexual activities were previously described among 46 MSM within this cohort (12).

Eleven of the 93 ES seroconverted, with an observed seroincidence rate of 3.29 HIV-1 infections per 100 person years (95% confidence interval [CI], 1.35, 5.24). All 11 seroconverters were MSM, which corresponds to 3.94 HIV-1 infections per 100 person years for all 76 MSM in the ES cohort (95% CI, 1.61, 6.26). We observed a lower seroincidence rate in the ES MSM in contrast to MSM reporting unprotected intercourse, regardless of partner HIV-1 serostatus, who enrolled in the Seattle HIVNET Vaccine Preparedness Study (7.68; 95% CI, 4.13, 14.27) (51). Thus, although the ES persistently engaged in high-risk sexual activities, there was a trend toward lower HIV-1 susceptibility during the period of our study than in the contemporaneous Seattle HIVNET MSM cohort.

Increased frequency of the CCR5 −2459 A/G genotype in CCR5 ORF Δ32/wt ES. We hypothesized that maintenance of HIV-1 seronegativity in our ES cohort could be attributed to an increased prevalence of protective genetic polymorphisms in HIV-1-binding chemokine receptors. To explore this, we compared the CCR5 promoter −2459G, the CCR5-Δ32, and the CCR2-64I allele frequencies in the 93 ES and a control population consisting of 247 persons reporting low-risk sexual activities. The overall CCR5-Δ32 allele frequencies were 14.5% in ES and 9.1% in the controls ($P = 0.041$; Table 2). This difference was attributable to the higher percentage of Caucasians in our ES cohort (96.7% versus 74.9% in the controls; $P < 0.0001$), particularly since the CCR5-Δ32 allele frequencies did not differ significantly between ES and controls when only Caucasians (15.0% versus 11.9%; $P = 0.308$) or Caucasian men (16.0% versus 14.4%; $P = 0.673$; Table 2) were compared. The CCR2-64I allele frequencies were similar in ES and controls (7.0% versus 9.1%; $P = 0.378$; Table 2), as well as when stratified by Caucasian ethnicity (6.1% versus 8.4%; $P = 0.348$) and Caucasian men (4.5% versus 7.2%; $P = 0.28$; Table 2).

The CCR5 −2459G allele frequencies did not differ significantly between ES and controls when all individuals (46.2% versus 46.4%; $P = 0.978$; Table 2) or all Caucasians (46.7% versus 42.2%; $P = 0.318$) were analyzed. However, a trend toward a higher frequency of the protective CCR5 −2459G allele in ES Caucasian men was observed (45.5% versus 37.5%; $P = 0.124$; Table 2). Since the majority of the ES were Caucasian men (84% versus 42% in the controls), we focused our subsequent analyses of CCR5 −2459 genotypes on Caucasian men from the ES and the control populations. The two genotypes containing one or two CCR5 −2459G alleles, −2459 A/G and −2459 G/G, were more frequent in ES than in control Caucasian men (74.4% versus 57.7%; $P = 0.02$; Table 3). This increase was reflected primarily by the predominance of the CCR5 −2459 A/G genotype among CCR5 Δ32/wt heterozygote ES subjects. In this case, 74% of Δ32/wt ES subjects carried the −2459 A/G genotype, compared to 37% of Δ32/wt controls ($P = 0.012$; Table 3). Of note, due to complete linkage disequilibrium between Δ32 and −2459A (35, 39), Δ32/wt heterozygotes cannot carry the −2459 G/G genotype. In contrast, among CCR5 wt/wt individuals, the CCR5 −2459 A/G and −2459 G/G genotypes were not significantly more frequent in ES subjects than in controls (78.6% versus 66.2%; $P = 0.122$; Table 3). Thus, in comparison to the controls, ES Caucasian

TABLE 2. Allele frequencies of CCR5 ORF wt/Δ32, CCR5 −2459
A/G, and CCR2 ORF 64V/I in ES and normal-risk
control individuals

Alleles	No. with allele					
	All genders and ethnicities			Caucasian men		
	ES ($n = 93$)	Control ($n = 247$)	P^a	ES ($n = 78$)	Control ($n = 104$)	P^a
CCR5 ORF						
wt	159	449	0.041	131	178	0.673
Δ32	27	45		25	30	
CCR2 ORF						
64V	173	449	0.378	149	193	0.28
64I	13	45		7	15	
CCR5 −2459						
A	100	265	0.978	85	130	0.124
G	86	229		71	78	

[a] Chi-square test.

TABLE 3. Genotype frequencies of CCR5 ORF Δ32 and CCR5 −2459 in Caucasian men

Genotype		No. with genotype		
CCR5 ORF	CCR5 −2459	ES (n = 78)	Control (n = 104)	P[a]
All	A/A	20	44	0.02
	A/G + G/G	58	60	
wt/wt	A/A	12	25	0.122
	A/G + G/G	44	49	
Δ32/wt	A/A[b]	5	19	0.012
	A/G[b]	14	11	
Δ32/Δ32	A/A[b]	3	0	NA[c]

[a] Chi-square test.
[b] Δ32 is in complete linkage disequilibrium with −2459A.
[c] NA, not applicable.

men who carried the CCR5-Δ32 allele on one chromosome were more likely to carry the CCR5 −2459G allele on the other chromosome. This observation suggests that the combined CCR5 ORF Δ32/wt plus −2459 A/G genotype confers an advantage in resisting HIV-1 infection.

Association of the combined CCR5 ORF Δ32/wt plus −2459 A/G genotype with lower CCR5 densities on circulating CD4+

T cells and CD14+ monocytes. The protective effect of the combined CCR5 ORF Δ32/wt plus −2459 A/G genotype may be due to lower expression levels of CCR5 on the surface of mucosal target cells encountered by HIV-1 during sexual transmission (18, 48). Because we were unable to obtain rectal or vaginal tissues in most of our study participants, we assessed PBMC as a surrogate to investigate this hypothesis. By flow cytometry, we determined the percentages of CCR5-expressing CD4+ T cells and CD14+ monocytes as well as their receptor surface densities in 71 ES subjects and 92 controls.

Among all genotype combinations, CCR5 ORF Δ32/wt plus −2459 A/G was associated with lower densities of CCR5 on CD4+ T cells (KW, $P = 0.0005$) and monocytes (KW, $P = 0.0206$) that expressed this receptor, but had no effect on the percentage of cells expressing CCR5 (Fig. 1). For CD4+ T cells, CCR5 densities ranged from 854 to 5,284 (median, 2,087) in Δ32/wt plus −2459 A/G individuals, compared to 1,589 to 6,654 (median, 4,296) in Δ32/wt plus −2459 A/A individuals (Mann-Whitney, $P = 0.026$; Dunn, $P > 0.05$) and 1,198 to 12,422 (median, 4,490) in all CCR5 ORF wt/wt individuals (Mann-Whitney, $P = 0.0001$; Dunn, $P < 0.001$). For CD14+ monocytes, CCR5 densities ranged from 2,948 to 12,266 (median, 5,293) in Δ32/wt plus −2459 A/G individuals, compared to 5,838 to 13,548 (median, 11,241) in Δ32/wt plus −2459 A/A individuals (Mann-Whitney, $P = 0.0113$; Dunn, $P < 0.05$) and
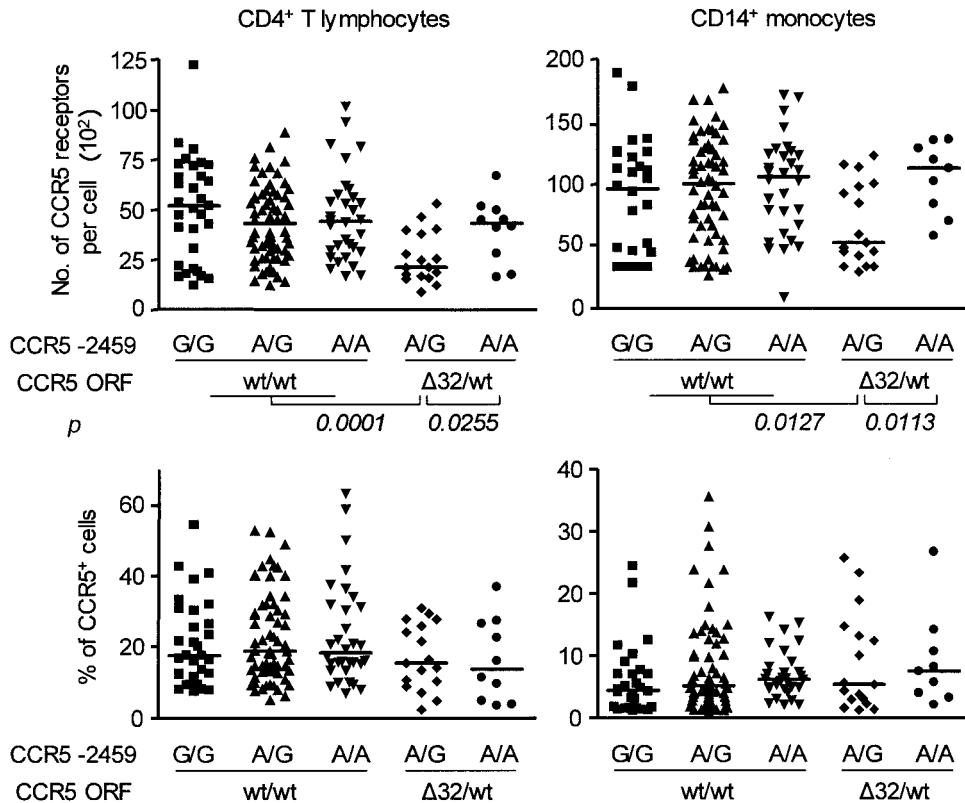

FIG. 1. Association between CCR5 ORF plus −2459 genotype combinations and CCR5 expression. Stored peripheral blood CD4+ T cells and CD14+ monocytes from 163 individuals were blinded and analyzed by FACS for the mean number of CCR5 surface receptors per CCR5+ cell and the percentage of CCR5-expressing cells. CCR5 genotypes were determined by restriction fragment length polymorphism analysis. Stratification of CCR5 densities and percentages by CCR5 ORF plus −2459 genotype combinations was performed after completion of all FACS analyses. Horizontal bars represent population medians. P values were determined by Mann-Whitney test. wt, wild type.
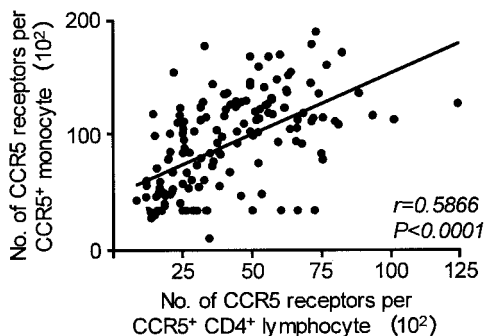
FIG. 2. Correlation between T-helper cell and monocyte surface CCR5 densities. Peripheral blood CD4$^+$ T cells and CD14$^+$ monocytes from 163 individuals were analyzed by FACS for the mean number of CCR5 surface receptors per CCR5$^+$ cell. Line fit was done by linear regression. r, Spearman correlation coefficient.

FIG. 3. HIV-1 infectivity of CD4$^+$ lymphoblasts. CD4-selected, PHA-stimulated lymphoblasts from 102 individuals were infected with HIV-1$_{JR-CSF}$ at MOI of 0.006 (not shown) and 0.003 (shown). p24 concentrations in culture supernatants were determined 7 days after infection. Stratification of p24 values by CCR5 genotype combinations was performed after completion of all infection assays. Numbers next to symbols are the mean number of CCR5 surface receptors per cell measured on peripheral blood CCR5$^+$ CD4$^+$ T cells from the same individuals but obtained at different clinic visits from those used to obtain the cells used for the infectivity assays. nd, not done.

918 to 18,863 (median, 10,182) in all CCR5 ORF wt/wt individuals (Mann-Whitney, $P = 0.0127$; Dunn, $P < 0.05$).

The CCR5 ORF Δ32/wt or −2459 A/G genotype individually had no significant effect on CCR5 densities on the HIV-1 target cells. Likewise, the CCR2-64I allele independently or in the various haplotypic combinations with the other two genomic loci had no influence on CCR5 expression (data not shown). The CCR5 densities on circulating CD4$^+$ T cells and monocytes were positively correlated (Spearman correlation, $r = 0.5866$; $P < 0.0001$; Fig. 2). We did not observe a trend toward higher or lower expression of CXCR4 in ES versus controls or between any of the genotypes, with the exception of negligible percentages and densities of CXCR4 on monocytes from Δ32/Δ32 persons (mean percentage of CXCR4$^+$ monocytes, 0.54%; $n = 3$). Thus, the combined CCR5 ORF Δ32/wt plus −2459 A/G genotype, present in increased frequencies in the ES Caucasian men, is associated with lower expression levels of CCR5 on both circulating CD4$^+$ T cells and CD14$^+$ monocytes.

Peripheral CD4$^+$ T lymphoblasts from ORF Δ32/wt plus −2459 A/G individuals do not exhibit increased resistance to direct in vitro infection. We postulated that the lower expression levels of CCR5 observed in CCR5 ORF Δ32/wt plus −2459 A/G individuals decreased the susceptibility of target cells to infection with R5-tropic HIV-1 strains (1, 18, 24, 44, 48). To screen for this effect, we infected PHA-stimulated CD4$^+$ T lymphoblasts from 77 ES and 25 control individuals with HIV-1$_{JR-CSF}$ and stratified infectivity levels by CCR5 ORF plus −2459 genotype combinations. In preliminary experiments in seven low-risk control donor cells, we determined MOI of 0.003 and 0.006 as optimal viral inocula to detect gradually increasing Gag p24 production over time in cell culture supernatants and with a common peak production at day 7 after infection. For both infecting doses, no overall difference in infectivity was observed between CCR5 ORF Δ32/wt plus −2459 A/G persons and persons harboring other genotype combinations (Fig. 3). Likewise, we did not observe a correlation between the infectivity of CD4$^+$ lymphoblasts and CCR5 densities on circulating T-helper cells obtained from the same individuals at different venipuncture dates (Spearman, $r = 0.1585$; $P = 0.1577$; data not shown). However, six donors had markedly lower infectivity levels than the others (Fig. 3). All six
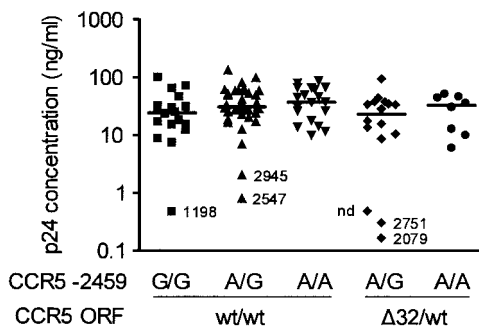
possessed at least one CCR5 −2459G allele, and three of the six (50%) harbored the CCR5 ORF Δ32/wt plus −2459 A/G genotype combination. Five of the six donor cells with lower infectivity were also tested for CCR5 expression densities on CD4$^+$ T cells, and these were all below the low 33rd percentile. In addition, lack of CCR5 expression in the three individuals homozygous for the CCR5-Δ32 allele correlated with absence of productive in vitro infection (data not shown). We conclude that the observed relative in vivo resistance to HIV-1 infection of the CCR5 ORF Δ32/wt plus −2459 A/G genotype and its associated decrease in CCR5 expression are not reflected by the in vitro infectivity of PHA-activated CD4$^+$ T lymphoblasts.

DISCUSSION

In contrast to CCR5-Δ32 homozygosity, which confers near 100% protection against HIV-1 infection (7, 26, 49), the benefit of carrying just one Δ32 allele has not been well defined. Published data demonstrate a disease-retarding effect of the CCR5 Δ32/wt genotype in HIV-1-infected individuals (5, 17, 38, 39, 45) but are conflicting as to whether Δ32 heterozygosity is associated with relative resistance to HIV-1 transmission (7, 13, 14, 31, 37, 49, 61). Our findings here demonstrate that a single Δ32 allele exerts a protective effect against viral transmission only if it occurs combined with the −2459G allele in the CCR5 promoter region (33, 35) on the other chromosome. Significantly lower CCR5 expression in these CCR5 Δ32/wt plus −2459 A/G individuals as compared to other genotype combinations suggests that decreased CCR5 expression is the factor limiting viral transmission. In contrast, CCR2 ORF 64I carrier status was not associated with resistance to infection, which is in keeping with previously published data (53), or with diminished CCR5 expression.

The observed enrichment of the CCR5 −2459 A/G genotype within our Δ32/wt Caucasian ES MSM also led to a significant increase of the overall frequency of the CCR5 Δ32/wt plus −2459 A/G genotype combination across the whole ES cohort when compared to all control individuals ($P = 0.0478$). This

was mostly attributable to the markedly increased percentage of Caucasians in our ES cohort (95% in ES versus 75% in controls), since the Δ32 allele is relatively common in Caucasians (~10%), but rare in other ethnicities (26, 28, 34, 49). It is possible that Caucasians became overrepresented in our ES cohort because they more often than other ethnicities display a relative resistance to infection. Our study shows that part of this ethnic advantage may be related to the increased prevalence of the CCR5 Δ32/wt plus −2459 A/G genotype combination in Caucasians compared to other ethnic groups. For example, among the 65 non-Caucasians in our two cohorts, only one Pacific Islander was a Δ32 heterozygote. Such a low frequency of the Δ32 allele in non-Caucasians is in agreement with other reports (26, 34, 49), and the CCR5 Δ32/wt plus −2459 A/G genotype combination therefore plays a miniscule role at best for resistance to infection in non-Caucasian cohorts, such as exposed seronegative female commercial sex workers in Africa (46).

An enrichment of the CCR5 Δ32/wt plus −2459 A/G combination would be expected in Caucasian ES versus Caucasian controls. We observed 17.9% CCR5 Δ32/wt plus −2459 A/G individuals in ES versus 10.6% in control Caucasians. Although this difference was not statistically significant ($P = 0.215$), when we compared our ES cohort to much larger Caucasian control cohorts that were reported by others, the enrichment of the CCR5 Δ32/wt plus −2459 A/G genotype combination in our ES cohort was apparent: Gonzales et al. (10), $n = 959$ and $P = 0.0211$; the Multicenter AIDS Cohort (MACS) results shown by Tang et al. (55), $n = 469$ and $P = 0.0829$; McDermott et al. (35) $n = 347$ and $P = 0.0199$; and the three control cohorts combined, $n = 1,775$ and $P = 0.0213$. Similar to our ES cohort, Tang et al. have reported a trend for an enrichment of the CCR5 Δ32/wt plus −2459 A/G genotype in the 90 most highly exposed and persistently HIV-1-seronegative men (HEPS) in the MACS cohort (55). If the HEPS and our ES cohort are combined ($n = 168$) and compared to the combined control cohorts, the level of significance increases further ($P = 0.0095$). This underscores that the CCR5 Δ32/wt plus −2459 A/G genotype combination is associated with a protective effect against HIV-1 transmission.

Both CCR5-Δ32 heterozygosity and the −2459G allele have been separately linked to lower CCR5 expression on PBMC (1, 2, 40, 48, 52, 57, 59), and the −2459G allele has also been shown to display lower CCR5 promoter activity by in vitro reporter gene analysis (35). In our study, CCR5-Δ32 heterozygosity or −2459G carrier status did not independently affect CCR5 expression. However, the combined CCR5 ORF Δ32/wt and CCR5 −2459 A/G genotype was clearly associated with decreased CCR5 densities on peripheral blood CD4$^+$ T cells and CD14$^+$ monocytes, indicating that both genotypes act cumulatively to restrict CCR5 expression. Of note, the highest CCR5 densities in the CCR5 Δ32/wt plus −2459 A/G group were around the median CCR5 expression measured for the other genotype combinations. This may reflect the finding by Tang et al. that CCR5 Δ32/wt plus −2459 A/G individuals could be further subdivided into two groups with distinct sets of additional genetic polymorphisms, only one of which was associated with resisting infection (55).

CCR5 densities on T cells and monocytes were positively correlated, indicating that trends toward high or low CCR5

expression existed across different cell types in each individual. This suggests that the relative CCR5 expression levels on peripheral blood CD4$^+$ T cells and CD14$^+$ monocytes may be extrapolated to leukocyte populations in other compartments. Thus, in CCR5 ORF Δ32/wt plus −2459 A/G individuals, HIV-1 transmission may be hindered by a relative paucity of CCR5 on mucosal target cells. Several studies have established a relationship between CCR5 cell surface density in various non-mucosal cell populations and susceptibility to infection (24, 40, 44, 45, 56, 60). It would be interesting to actually confirm that mucosal target cells such as intraepithelial Langerhans cells (LCs) express lower CCR5 levels and are less susceptible to infection in CCR5 ORF Δ32/wt plus −2459 A/G individuals. In an elegant study, Kawamura et al. have reported that skin-derived LCs from ORF Δ32/wt plus −2459 A/G individuals were markedly less susceptible to HIV-1 than LCs from ORF Δ32/wt plus −2459 A/A individuals (18). This finding most likely reflects a difference in CCR5 expression between ORF Δ32/wt −2459 A/G and −2459 A/A individuals—as we have seen in our cohorts—although the authors did not evaluate the CCR5 densities of the isolated LCs. Arguably, cell numbers obtained from mucosal or skin tissue samples are mostly too low to perform both receptor density and infectivity studies. Our infectivity experiments with CD4$^+$ lymphoblasts, however, indicate that results obtained with surrogate target cells need to be interpreted with caution. Using PHA-stimulated peripheral blood CD4$^+$ T lymphocytes for screening, we could not detect a difference in infectivity levels between ORF Δ32/wt −2459 A/G and −2459 A/A individuals. It is likely that in vitro hyperactivation overcame the differences that existed in CCR5 promoter activity before stimulation with the mitogen, and infectivity levels in the two groups were therefore equal. This finding also emphasizes the possibility that relative resistance to infection conferred by specific CCR5 genotype combinations can be overcome by cellular activation, as may be the case during sexually transmitted disease-triggered mucosal inflammation (15, 32, 42, 47).

In conclusion, our results indicate that some individuals who practice high-risk sexual behavior may resist infection because they possess the CCR5 Δ32/wt plus −2459 A/G genotype combination, which is associated with relatively weak CCR5 expression. This suggests that a single Δ32 allele can exert a protective effect against viral transmission only if it pairs with the −2459G allele in the CCR5 promoter region of the other chromosome. Despite the enrichment of the CCR5 Δ32/wt plus −2459 A/G genotype combination in our ES cohort, the majority of ES individuals do not carry it and additional mechanisms of resistance, such as the dose of CCR5-binding immune response genes (11), therefore have to be considered.

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