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Research Article

HIV-1-infected long-term nonprogressors are a heterogeneous group of individuals with regard to immunologic and virologic markers of HIV-1 disease. CC chemokine receptor 5 (CCR5) has recently been identified as an important coreceptor for HIV-1 entry into CD4+ T cells. A mutant allele of CCR5 confers a high degree of resistance to HIV-1 infection in homozygous individuals and partial protection against HIV disease progression in heterozygotes. The frequency of CCR5 heterozygotes is increased among HIV-1-infected long-term nonprogressors compared with progressors; however, the host defense mechanisms responsible for nonprogression in CCR5 heterozygotes are unknown. We hypothesized that nonprogressors who were heterozygous for the mutant CCR5 gene might define a subgroup of nonprogressors with higher CD4+ T cell counts and lower viral load compared with CCR5 wild-type nonprogressors. However, in a cohort of 33 HIV-1-infected long-term nonprogressors, those who were heterozygous for the mutant CCR5 gene were indistinguishable from CCR5 wild-type nonprogressors with regard to all measured immunologic and virologic parameters. Although epidemiologic data support a role for the mutant CCR5 allele in the determination of the state of long-term nonprogression in some HIV-1-infected individuals, it is not the only determinant. Furthermore, long-term nonprogressors with the wild-type CCR5 genotype are indistinguishable from heterozygotes from an immunologic and virologic standpoint.

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Heterozygosity for a Defective Gene for CC Chemokine Receptor 5 Is Not the Sole Determinant for the Immunologic and Virologic Phenotype of HIV-infected Long-Term Nonprogressors

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Abstract

HIV-1-infected long-term nonprogressors are a heterogeneous group of individuals with regard to immunologic and virologic markers of HIV-1 disease. CC chemokine receptor 5 (CCR5) has recently been identified as an important coreceptor for HIV-1 entry into CD4+ T cells. A mutant allele of CCR5 confers a high degree of resistance to HIV-1 infection in homozygous individuals and partial protection against HIV disease progression in heterozygotes. The frequency of CCR5 heterozygotes is increased among HIV-1-infected long-term nonprogressors compared with progressors; however, the host defense mechanisms responsible for nonprogression in CCR5 heterozygotes are unknown. We hypothesized that nonprogressors who were heterozygous for the mutant CCR5 gene might define a subgroup of nonprogressors with higher CD4+ T cell counts and lower viral load compared with CCR5 wild-type nonprogressors. However, in a cohort of 33 HIV-1-infected long-term nonprogressors, those who were heterozygous for the mutant CCR5 gene were indistinguishable from CCR5 wild-type nonprogressors with regard to all measured immunologic and virologic parameters. Although epidemiologic data support a role for the mutant CCR5 allele in the determination of the state of long-term nonprogression in some HIV-1-infected individuals, it is not the only determinant. Furthermore, long-term nonprogressors with the wild-type CCR5 genotype are indistinguishable from heterozygotes from an

immunologic and virologic standpoint. (*J. Clin. Invest.* 1997. 100:1581–1589.) Key words: HIV-1 • disease progression • CC chemokine receptor 5 • polymorphism • lymph nodes

Introduction

The natural history of infection with HIV is highly variable and dependent on multiple viral and host factors (1, 2). A small percentage of individuals infected with HIV-1 for long periods of time remain asymptomatic and free from progressive immunodeficiency; these individuals are referred to as long-term nonprogressors (3–7). Compared with individuals with progressive HIV-1 disease, long-term nonprogressors typically have lower viral load, more vigorous HIV-1-specific cell-mediated immune responses, and higher titers of HIV-1-neutralizing antibodies (3–8). In addition, lymph nodes from long-term nonprogressors do not exhibit the typical architectural disruptions associated with progressive HIV-1 disease (6). Although evidence of viral attenuation is present only in the minority of nonprogressors (9–12), a high degree of viral diversity is present, apparently driven by vigorous cell-mediated immune responses (13, 14). Despite these clear-cut differences compared with progressors, considerable variability is observed in the aforementioned parameters among long-term nonprogressors, and it is clear that they constitute a heterogeneous group.

CC chemokine receptor 5 (CCR5),¹ a seven-transmembrane G protein-coupled cellular receptor, has recently been shown to be a coreceptor for macrophage (M)-tropic or non-syncytium-inducing (NSI) strains of HIV-1 (15–19), which are the predominant transmitting strains of the virus (20, 21). Natural ligands for CCR5 include macrophage inflammatory pro-

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tein (MIP)-1 α , MIP-1 β , and RANTES (regulated-upon-activation, normal T expressed and secreted), which have been shown to inhibit HIV replication in CD4+ T cells and are secreted by a variety of cell types (22), including CD8+ T cells (23). Studies of individuals who have been multiply-exposed to HIV-1 yet remain uninfected (exposed-uninfected), suggested that CD8+ T cells from these individuals secrete high levels of MIP-1 α , MIP-1 β , and RANTES (24). However, a role for CCR5 in the pathogenesis of HIV-1 disease was demonstrated when two exposed-uninfected individuals were found to be homozygous for a 32-bp deletion within the CCR5 open reading frame (CCR5- Δ 32), which yields a truncated, nonfunctional protein (25).

Several lines of evidence have suggested that homozygosity for the inactivating mutation within CCR5 confers resistance to HIV-1 infection. In this regard, molecular epidemiologic studies have identified very few CCR5- Δ 32 homozygotes among thousands of HIV-1-infected individuals, despite the fact that homozygosity confers no overt deleterious phenotype and is found in \sim 1% of the HIV-1-uninfected Caucasian population (26–32). In addition, the CCR5- Δ 32 homozygous genotype is enriched in populations of exposed-uninfected individuals (27–29). Finally, PBMC from CCR5- Δ 32 homozygotes fail to support fusion with M-tropic HIV-1 envelope-expressing cells, and are resistant to infection with M-tropic strains of HIV-1 (25, 26).

Several groups have shown that cohorts of long-term non-progressors are enriched for individuals heterozygous for the mutant CCR5 gene, and that heterozygous seroconverters experience a slower rate of disease progression compared with CCR5 wild-type seroconverters (27, 29, 33, 34). These findings suggest that while CCR5- Δ 32 homozygotes may be protected from HIV-1 infection, heterozygotes may be partially protected against disease progression. The preliminary observation that CD4+ T cells from CCR5- Δ 32 heterozygotes support viral entry and replication with decreased efficiency compared with cells from CCR5 wild-type individuals suggested that lower viral load and slower loss of CD4+ T cells might be found in these compared with CCR5 wild-type individuals (25). Therefore, we examined immunologic and virologic parameters of HIV-1 disease progression in a cohort of 33 long-term non-progressors in order to determine whether CCR5- Δ 32 heterozygotes constitute a distinct subgroup of non-progressors with regard to CD4+ T cell counts and viral loads compared with CCR5 wild-type non-progressors.

Methods

Patients. 33 individuals with long-term nonprogressive HIV-1 infection were studied at the National Institutes of Health Clinical Center. Criteria for the definition of long-term nonprogression included documented HIV-1 seropositivity for longer than 7 yr, stable CD4+ T cell counts $> 600/\text{mm}^3$, absence of symptoms, and no history of antiretroviral treatment (6). Immunologic and virologic characteristics of 9 of the 33 subjects have been reported previously (6). The mean age of the subjects was 42.2 yr. 31 subjects (93.9%) were males who had contracted HIV-1 infection through homosexual contact. There were 2 females among the non-progressors: one had contracted HIV-1 infection through heterosexual contact, and one had no known risk factor for HIV infection. 29 (87.9%) of the subjects were Caucasian, and 4 (12.1%) were African-American. The mean duration of documented HIV-1 seropositivity was 11.0 yr; the mean CD4+ T cell count was

897 cells/mm 3 ; and the geometric mean level of plasma viremia was 2,065 HIV-1 RNA copies/ml of plasma (see Table I).

Lymph node biopsies. Excisional lymph node biopsies were performed on 26 of the 33 long-term nonprogressors according to a protocol approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases.

Genotypic analysis of CCR5. Genotypic analysis of CCR5 alleles was performed as described, and the results were reported previously in preliminary form (29). The full-length open reading frame of CCR5 was amplified from genomic DNA (sense primer, 5' CCC-AAGCTTACTCTCCCCGGGTGGAACAAAGATG 3'; antisense primer, 5' GGTCTAGAGTCACCAGCCCACITGAGTCCTGTG 3') by PCR. CCR5 PCR products were subjected directly to BglII digestion, and the fragments were visualized after electrophoresis with ethidium bromide staining. A BglII restriction site is located upstream of the 32-bp deletion site in the CCR5 gene such that the 3' half of BglII-digested CCR5 yields a 582-bp product in wild-type homozygotes, a 582- and a 550-bp product in CCR5- Δ 32 heterozygotes, and only a 550-bp product in CCR5- Δ 32 homozygotes.

Analysis of surface expression of CCR5. PBMC were stained in a solution of 2% BSA in Dulbecco's phosphate-buffered saline (dPBS) containing 0.1% azide with 10 $\mu\text{g}/\text{ml}$ anti-CCR5 (clone 2D7-13; a generous gift from Dr. Charles Mackay, Leukocite, Inc., Boston, MA), 10 $\mu\text{g}/\text{ml}$ anti-CCR3 (clone 7B11; also a gift from Dr. Mackay), 8 $\mu\text{g}/\text{ml}$ anti-CXCR4 (clone 12G5; a generous gift from Dr. James Hoxie, University of Pennsylvania, Philadelphia, PA), or an isotype-matched control antibody for 30 min at 4°C. Cells were then washed in 2% BSA/dPBS, resuspended, and stained with an FITC-conjugated goat anti-mouse F(ab') $_2$ antibody at 1:100 final dilution for 15 min at 4°C. Cells were washed again in 2% BSA/dPBS and incubated with a blocking solution of 1 $\mu\text{g}/\text{ml}$ purified mouse Ig (Sigma Chemical Co., St. Louis, MO) for 10 min at 4°C. After another wash with 2% BSA/dPBS, cells were stained with phycoerythrin-conjugated anti-CD4 antibody (Becton Dickinson, San Jose, CA) for 15 min at 4°C. Cells were then washed twice with 2% BSA/dPBS, resuspended in 0.3 ml of 2% BSA/dPBS, and analyzed with a flow cytometer (model XL; Coulter Corp., Miami, FL). Forward and orthogonal light scatter gates were drawn to include lymphocytes and monocytes. These gated events were further analyzed for the presence of chemokine receptors CCR5, CCR3, or CXCR4 on CD4+ cells.

Analysis of in vitro responses of PBMC to various stimuli. Fresh PBMC (10^5 cells in 200 μl) were placed in 96-well round-bottomed microtiter plates. The culture medium consisted of RPMI 1640 with 15% human AB serum. Stimuli included PHA 2 $\mu\text{g}/\text{ml}$ (Wellcome Industries, Beckenham, UK), alloantigen (50,000 irradiated allogeneic PBMC), and tetanus toxoid 5 $\mu\text{g}/\text{ml}$ (Connaught Laboratories, Swiftwater, PA). Proliferative responses were measured by incorporation of [^3H]thymidine (New England Nuclear, Boston, MA) over a 4-h pulse with 0.44 μCi of [^3H]thymidine. Responses to PHA were measured on day 3 of culture, to alloantigens on day 5, and to tetanus toxoid on day 6. The stimulation index was calculated as the counts per minute (cpm) for each stimulus minus the background cpm (unstimulated cells), divided by the background cpm. A stimulation index > 5 was considered a positive response.

Determination of plasma viremia by the branched-chain DNA (bDNA) assay. HIV-1 RNA was quantitated in plasma using the Quantiplex® bDNA assay kit according to the manufacturer's instructions (Chiron Corporation, Emeryville, CA). The concentration of RNA in each specimen is expressed as HIV-1 RNA copies per milliliter, determined from a standard curve using a DNA plasmid at four concentrations. The dynamic range of the assay is 500–800,000 RNA copies/ml of plasma, and the sensitivity is 500 HIV-1 RNA copies/ml. Measurements < 500 HIV-1 RNA copies/ml were considered equal to 500 HIV-1 copies/ml for the purposes of calculations for statistical analysis.

Analysis of HIV-1 DNA in PBMC and lymph node mononuclear cells (LNMC) by PCR. Proviral HIV-1 DNA was quantitated by a semiquantitative PCR assay (35) with a primer pair (SK145/101) spe-

cific for the gag gene segment. 32 PBMC specimens and 24 LNMC specimens were available for analysis. Results are expressed as HIV-1 DNA copies per million cells.

Analysis of HIV-1 replication in LNMC by reverse transcription-PCR. Total RNA was extracted from LNMC from 18 nonprogressors, and 2 µg was reverse-transcribed with random hexamers as described previously (36). One-tenth of each reverse transcription was amplified with a common sense primer (5' GACTCATCAAGCT-TCTCTATCAAA 3') corresponding to a sequence within the first tat/rev exon and an antisense primer which detects either unspliced plus single-spliced HIV-1 RNA (complementary to a sequence within env: 5' CTCATTGCCACTGTCTTCTGCTCT 3') or multiply-spliced HIV-1 RNA (complementary to a sequence within the second tat/rev exon: 5' TCTCAAGCGGTGGTAGCTGA 3') (36). Positive controls for the detection of unspliced plus single-spliced HIV-1 RNA were serial dilutions of a plasmid cDNA construct containing HIV-1 exons 1 and 4E; for the detection of multiply-spliced HIV-1 RNA, serial dilutions of a plasmid construct containing HIV-1 exons 1, 5, and 7 served as positive controls (37). PhosphorImager analysis was used for signal quantitation.

To normalize for RNA input, 1/25 of each reverse transcription was amplified with a primer pair specific for human T cell receptor Cα mRNA (36). Quantitation of HIV-1 RNA was accomplished by normalization of the HIV-1 amplification signal to the Cα signal and interpolation into the control cDNA serial dilution curve.

In situ hybridization, and immunohistochemical and morphometric analysis of lymphoid tissue. In situ hybridization was performed with a mixture of ³⁵S-radiolabeled RNA probes synthesized from five DNA templates that cumulatively represent 90% of the HIV-1 genome (38). Immunohistochemical analysis was performed with anti-CD21 antibody (DAKO Corp., Carpinteria, CA), which stains follicular dendritic cells in formaldehyde-fixed, paraffin-embedded tissues. Morphometric analysis of the percentage of lymph node area occupied by either CD21 immunostain ($n = 24$ of the 26 lymph nodes biopsied) or by HIV-1 RNA detected by in situ hybridization ($n = 22$) was performed with a videoscope camera (CCD 200E; Videoscope International, Reston, VA) and video-planimetry computer software based on National Institutes of Health Image 1.60. Productively infected cells detected by in situ hybridization were counted manually and expressed as the number of productively infected cells per 10 square millimeters of lymph node tissue ($n = 25$).

Determination of serum levels of β-chemokines. Serum levels of RANTES, MIP-1α, and MIP-1β were determined by enzyme immunoassay according the manufacturer's instructions (R & D Systems, Inc., Minneapolis, MN). 24 serum samples were available for this analysis.

Statistical analysis. Mean values for measured variables are reported \pm SE. Geometric mean values with 95% confidence intervals (CI) are reported for logarithmic normally distributed variables (i.e., plasma viremia and HIV-1 proviral burden). Comparisons of the proportion of CCR5-Δ32 heterozygotes in different groups as well as the proportion of CCR5 wild-type and CCR5-Δ32 heterozygous individuals whose PBMC responded in vitro to stimuli were made by χ^2 analysis.

The molecular epidemiologic analysis of CCR5 genotypes was limited to Caucasians ($n = 29$ among the long-term nonprogressors) because of the very low frequency of the CCR5-Δ32 allele in nonCaucasian populations (26, 29). Comparison of measured variables between the CCR5 wild-type and CCR5-Δ32 heterozygote group was made by the Mann-Whitney U test: these comparisons were made within the entire cohort of 33 long-term nonprogressors.

Results

CCR5 genotype. We have reported recently that among randomly selected North American Caucasian blood donors ($n = 387$), 77.5% were CCR5 wild-type, 21.7% were CCR5-Δ32

Table I. Demographic, Immunologic, and Virologic Characteristics of HIV-infected Long-Term Nonprogressors Stratified by CCR5 Genotype

Patient	Age	Years HIV+	CD4+ T cell count*	CD8+ T cell count*	Plasma viremia [†]
Wild-type CCR5 genotype					
001	42	12	810	870	23000
002	34	7	992	970	500
003	45	13	720	2483	500
004	41	12	612	1023	26000
005	44	11	972	1549	500
006	36	11	636	562	2708
007	50	10	766	867	97000
008	34	11	1158	1129	6000
009	43	12	1053	2379	500
010	35	11	890	554	1000
011	31	10	653	1110	1000
012	46	10	1633	1750	500
013	45	11	1238	2090	500
014	48	9	1038	1291	500
015	35	12	1287	1141	669
016	39	11	767	2013	2000
017	38	11	637	1274	4757
018	47	8	938	1488	37000
019	55	11	790	909	500
020	37	11	607	805	14210
021	49	11	908	739	500
Mean	41.6 \pm 1.4	10.7 \pm 0.3	910 \pm 57	1286 \pm 124	2104 (992–4460)
Heterozygous CCR5 genotype					
022	37	12	817	1010	3000
023	50	7	1043	844	620
024	33	8	1066	1904	3000
025	56	18	646	2926	36000
026	35	11	605	549	5000
027	42	10	1026	879	500
028	36	10	675	527	7000
029	42	13	690	858	500
030	49	9	1450	673	500
031	49	13	685	1178	18000
032	43	16	773	855	500
033	45	12	1142	961	500
Mean	43.1 \pm 2.0	11.6 \pm 0.9	885 \pm 75	1097 \pm 196	1995 (824–4832)
<i>P</i> values (wild-type vs. heterozygous CCR5 genotype)					
	0.57	0.50	0.88	0.12	0.99

*Cells/mm³. [†]HIV-1 RNA copies/ml. Values which were below the sensitivity limit of the assay (i.e., 500 copies/ml) were considered equal to 500 HIV-1 RNA copies/ml for the purposes of statistical analysis. Mean values are presented \pm SE, except in the case of plasma viremia, for which the geometric mean values are presented with 95% CI.

heterozygous, and 0.8% were CCR5-Δ32 homozygous (29). Corresponding percentages among HIV-1-infected North American Caucasians ($n = 614$) were 77.4% CCR5 wild-type, 22.6% CCR5-Δ32 heterozygous, and 0% CCR5-Δ32 homozygous (29). These results are similar to those reported by other groups (27, 28). Among the 29 North American Caucasian

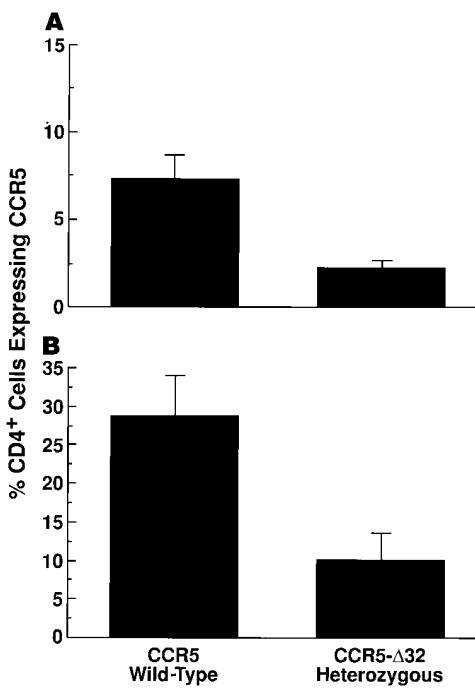


Figure 1. Comparative analysis of CCR5 expression on CD4+ lymphocytes (A) and monocytes (B) from CCR5 wild-type and CCR5-Δ32 heterozygous nonprogressors. A significantly higher percentage of CD4+ lymphocytes and monocytes expressed CCR5 from CCR5 wild-type compared with CCR5-Δ32 heterozygous nonprogressors ($P = 0.04$ and 0.008 , respectively). Mean values \pm SE are shown.

long-term nonprogressors in our cohort, 11 (37.9%) were CCR5-Δ32 heterozygotes (29), and this 68% increase in the expected frequency of CCR5-Δ32 heterozygotes compared with a broad cross-section of HIV-1-infected individuals ($n = 614$) was statistically significant ($P = 0.05$). There were no significant differences in the mean duration of documented HIV-1 seropositivity or the mean age of the CCR5 wild-type versus heterozygous long-term nonprogressors (Table I).

Cell surface expression of HIV coreceptors. Cell surface expression of CCR5 was significantly lower on CD4+ lymphocytes and monocytes from CCR5-Δ32 heterozygous long-term nonprogressors compared with CCR5 wild-type nonprogres-

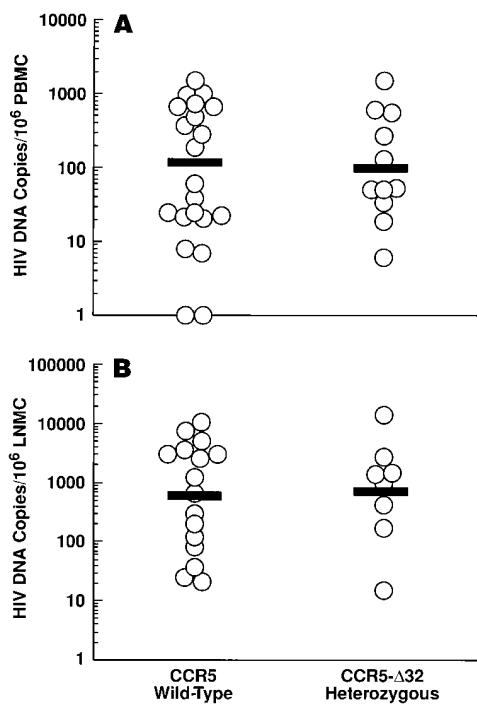
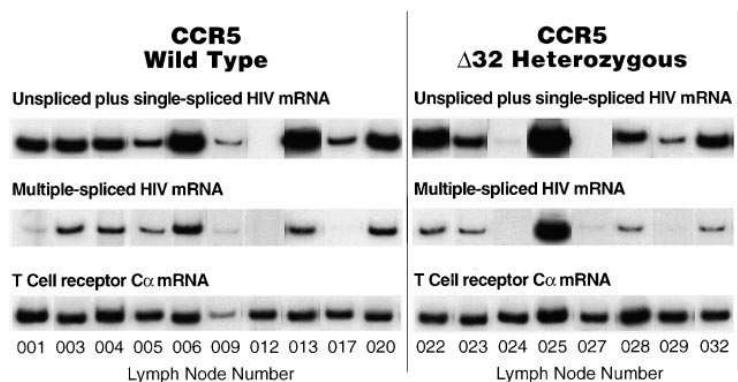


Figure 2. Comparative analysis of HIV-1 proviral burden in PBMC (A) and LNMC (B) from CCR5 wild-type and CCR5-Δ32 heterozygous long-term nonprogressors. Geometric mean values are indicated by horizontal bars. No significant differences in proviral burden were detected between the two groups of nonprogressors. Consistent with previous reports, the proviral burden in LNMC exceeded that in PBMC approximately sevenfold (35).

sors (Fig. 1). There was no significant difference between CCR5-Δ32 heterozygotes and CCR5 wild-type nonprogressors in CD4+ lymphocyte and monocyte expression of CXCR4 and CCR3 (data not shown).

Lymphocyte subsets. The mean CD4+ and CD8+ T cell counts were indistinguishable when CCR5 wild-type and heterozygous long-term nonprogressors were compared (Table I).

In vitro responses of PBMC to stimuli. PBMC from all of the long-term nonprogressors responded in vitro to PHA and to alloantigen (data not shown). PBMC from 10 of 21 (47.6%) CCR5 wild-type nonprogressors responded in vitro to tetanus



Quantitation of the PCR signals by phosphorImager analysis and normalization according to the Cα signal are described in Methods. A high degree of variability in the levels of HIV replication is seen in both CCR5 wild-type and CCR5-Δ32 heterozygous nonprogressors.

Figure 3. HIV replication detected by PCR in LNMC from CCR5 wild-type ($n = 10$, left) and CCR5-Δ32 heterozygous ($n = 8$, middle) long-term nonprogressors. Amplifications of unspliced plus single-spliced HIV RNA are shown (top row), including a dilutional series of a control plasmid (right). Amplifications of the same lymph node samples for multiply-spliced HIV RNA are shown (middle row) along with a dilutional series of a control plasmid (right). The bottom row shows amplifications of the same lymph node samples for T cell receptor Cα mRNA as a control for the relative total amount of RNA present.

toxoid compared with 7 of 12 (58.3%) CCR5-Δ32 heterozygotes ($P > 0.5$).

Plasma viremia. The geometric mean levels of plasma viremia were nearly identical in CCR5 wild-type and heterozygous nonprogressors (Table I). Potential confounding factors were the sensitivity limit of 500 HIV-1 RNA copies/ml detected by the bDNA assay, and the lower degree of reproducibility of the assay at low values. However, even if the five measurements of plasma viremia which were < 500 HIV-1 RNA copies/ml among the CCR5-Δ32 heterozygotes were considered to equal zero, and the nine values < 500 HIV-1 copies/ml among the CCR5 wild-type individuals were considered to equal 500 HIV-1 copies/ml, the mean values remained indistinguishable between the groups ($P = 0.41$).

HIV-1 proviral burden. Geometric mean HIV-1 proviral burden in PBMC was 120 (95% CI 44.7–322) HIV-1 DNA copies/million cells in CCR5 wild-type individuals and 100 (95% CI 37.0–267) in CCR5-Δ32 heterozygotes (Fig. 2; $P = 0.75$). Proviral burden in LNMC was 604 (95% CI 214–1,705) HIV-1 DNA copies/million cells in CCR5 wild-type individuals and 729 (95% CI 178–2,980) in CCR5-Δ32 heterozygotes (Fig. 2; $P = 0.99$).

HIV-1 replication in LNMC. Levels of viral replication in PBMC were generally at or below the limit of detectability (data not shown). There was no difference in the level of viral replication in LNMC from CCR5 wild-type nonprogressors compared with CCR5-Δ32 heterozygotes. The mean level of unspliced plus single-spliced HIV-1 mRNA was 22.9 ± 6.2 fg per 0.2 μ g total RNA in LNMC from CCR5 wild-type nonprogressors and 20.4 ± 8.4 in LNMC from CCR5-Δ32 heterozygous nonprogressors (Fig. 3; $P = 0.37$). The mean level of multiply-spliced HIV-1 mRNA in LNMC was 7.8 ± 1.8 fg per 0.2 μ g total RNA from CCR5 wild-type nonprogressors and 10.5 ± 6.0 from CCR5-Δ32 heterozygous nonprogressors (Fig. 3; $P = 0.66$).

Morphometric analysis of follicular hyperplasia, HIV-1 trapping, and viral expression in lymphoid tissue. The mean percentages of lymph node area occupied by CD21 immunostain (i.e., the relative degree of follicular hyperplasia) and HIV-1 RNA detected by *in situ* hybridization (i.e., the degree of viral trapping within germinal centers) were comparable between CCR5 wild-type and heterozygous long-term nonprogressors (Table II, and Fig. 4). There was also no significant difference in the mean number of productively infected cells per unit area of lymph node tissue between CCR5 wild-type and heterozygous nonprogressors (Table II).

Serum levels of β -chemokines. The mean serum levels of RANTES, MIP-1 α , and MIP-1 β among CCR5 wild-type nonprogressors were 43.9 ± 4.6 ng/ml, 214 ± 119 pg/ml, and 129 ± 14 pg/ml, respectively. Among CCR5-Δ32 heterozygous nonprogressors, mean serum levels of RANTES, MIP-1 α , and MIP-1 β were 40.9 ± 5.9 ng/ml ($P = 0.93$ compared with CCR5 wild-type nonprogressors), 42.9 ± 1.4 pg/ml ($P = 0.09$), and 110 ± 6 pg/ml ($P = 0.22$), respectively (Fig. 5).

Discussion

Recent studies have clearly demonstrated that individuals who are homozygous for the mutant CCR5 allele, which contains an inactivating 32-bp deletion in the open reading frame, are highly resistant to HIV-1 infection (25–29). Furthermore, the frequency of CCR5-Δ32 heterozygotes is increased among

Table II. Analysis of Lymph Node Germinal Center Area, Viral Trapping, and Number of Productively Infected Cells

Patient	CD21%*	HIV%‡	Productively infected cells§
Wild-type CCR5 genotype			
001	12.21	4.30	4.50
003	27.83	15.73	4.30
004	4.31	0.36	3.60
005	7.92	n.d.	2.60
006	10.55	4.58	4.80
008	22.67	2.33	7.80
009	5.86	1.13	4.50
010	13.00	1.80	10.20
011	11.82	1.02	1.70
012	n.d.	2.28	22.80
013	19.75	6.43	3.10
014	7.75	n.d.	0.50
015	17.74	3.45	12.30
017	11.28	4.24	0.40
020	25.40	4.85	9.80
021	7.47	n.d.	0.00
Mean±SE	13.70 ± 1.89	4.04 ± 1.09	5.81 ± 1.45
Heterozygous CCR5 genotype			
022	18.02	9.46	1.90
023	5.99	1.35	8.60
024	8.95	n.d.	1.10
025	12.65	5.00	8.10
026	8.59	13.48	1.80
028	10.66	3.07	9.90
029	5.79	1.32	3.30
031	10.88	5.61	9.60
032	9.52	0.44	0.50
Mean±SE	10.12 ± 1.23	4.97 ± 1.60	4.98 ± 1.32
<i>P</i> values (wild-type vs. heterozygous CCR5 genotype)			
	0.30	0.61	0.84

Studies done on 25 lymph node specimens available. *Percentage of lymph node area occupied by CD21 immunostain (i.e., relative germinal center area). ‡Percentage of lymph node area occupied by HIV RNA detected by *in situ* hybridization (i.e., degree of viral trapping in germinal centers). §Number of productively infected cells, detected by *in situ* hybridization per 10 mm² lymph node. ND, Not done.

HIV-infected long-term nonprogressors (29, 33, 34) and slow progressors (27). Based upon this observation as well as reported evidence of decreased efficiency of HIV-1 entry and replication in CD4+ T cells from CCR5-Δ32 heterozygotes (25, 39), we tested the hypothesis that within a group of HIV-infected long-term nonprogressors, CCR5-Δ32 heterozygotes might harbor lower viral loads and higher CD4+ T cell counts compared with CCR5 wild-type individuals. In agreement with the study by Wu et al. (39), we found that a significantly lower percentage of CD4+ cells from CCR5-Δ32 heterozygous nonprogressors coexpressed CCR5 compared with CD4+ cells from CCR5 wild-type nonprogressors. We demonstrate considerable heterogeneity within our cohort of long-term nonprogressors with regard to CD4+ and CD8+ T cell counts,

plasma viremia, viral burden in peripheral blood and lymph nodes, levels of viral replication in lymph nodes, the degree of follicular hyperplasia and viral trapping in germinal centers, and serum levels of the β -chemokines. However, heterogeneity with regard to these immunologic and virologic parameters did not diminish when the cohort was stratified according to CCR5 genotype.

It should be noted that although the measured immunologic and virologic parameters were indistinguishable between the two groups of nonprogressors defined by CCR5 genotype, it is possible that the limited sample size prevented the detection of real differences. Another caveat with regard to studies of long-term nonprogressors is the operational definition of nonprogression. We used a clinical definition of nonpro-

gression adapted from the Multicenter AIDS Cohort Study, wherein < 5% of HIV-infected individuals meet the nonprogressor criteria. Given recent data regarding the strong association between levels of plasma viremia and prognosis in HIV infection (40), we performed a subgroup analysis in this study which included only the 16 CCR5 wild-type and 10 CCR5- Δ 32 heterozygous nonprogressors with levels of plasma viremia < 10,000 HIV RNA copies/ml. This stringent analysis of immunologic and virologic parameters in long-term nonprogressors stratified by CCR5 genotype yielded results which were virtually identical to the analysis conducted in the entire cohort: CCR5 wild-type and CCR5- Δ 32 heterozygous nonprogressors remained immunologically and virologically indistinguishable.

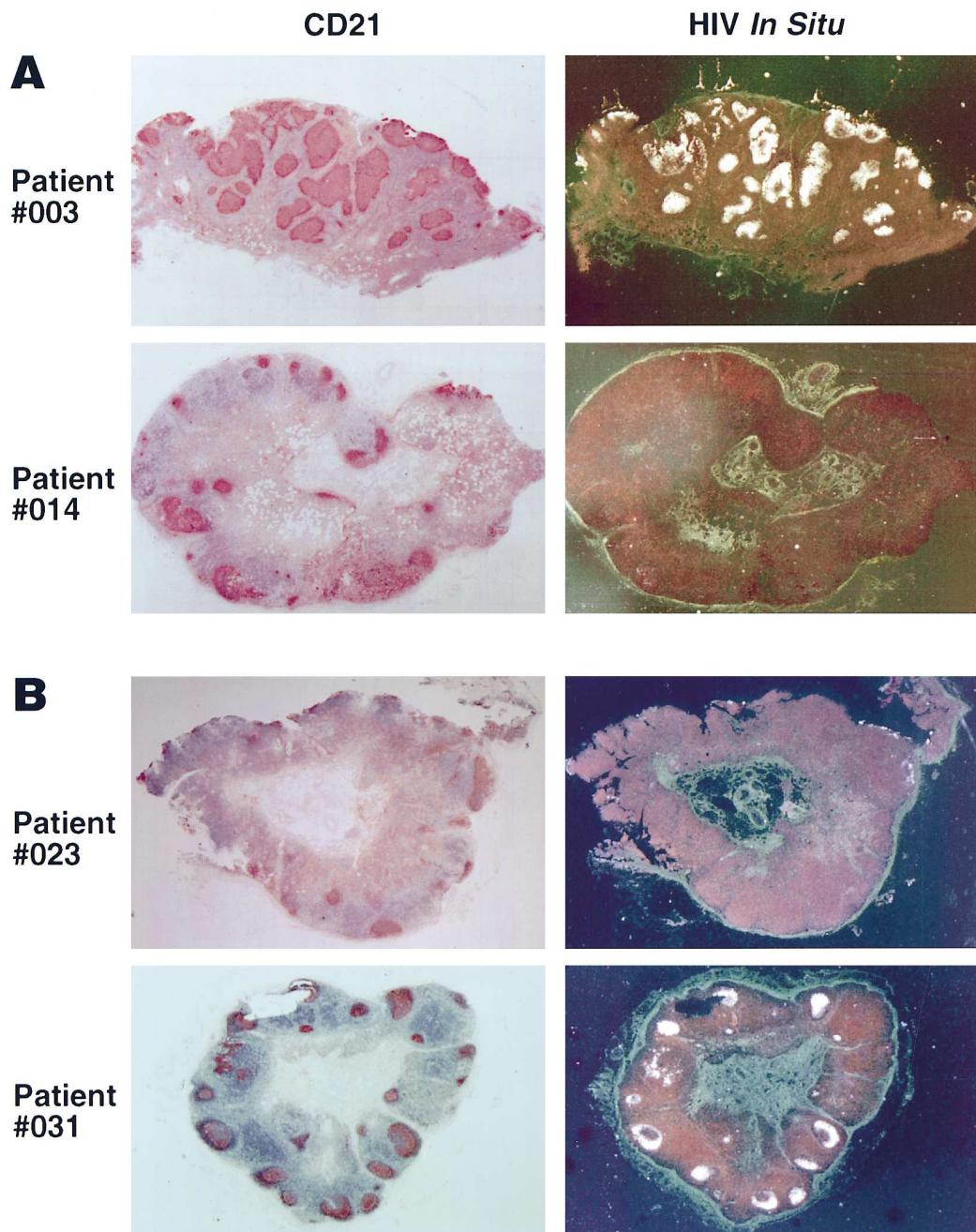


Figure 4. Distribution of HIV-1 RNA in lymph nodes of HIV-infected CCR5 wild-type and CCR5- Δ 32 heterozygous nonprogressors. Photomicrographs ($\times 35$) of immunohistochemistry for CD21 and in situ hybridization for HIV-1 RNA are shown in representative lymph node sections from CCR5 wild-type (A) and CCR5- Δ 32 heterozygous nonprogressors (B). CD21 staining, highlighting germinal centers, appears red (left column). HIV-1 RNA appears white in the dark-field micrographs (right column).

The failure of HIV-infected CCR5-Δ32 heterozygotes to constitute a distinct subgroup of nonprogressors with lower viral load and higher CD4+ T cell counts does not exclude a role for the mutant allele in protecting against disease progression. Epidemiologic data support a partially protective role against disease progression for CCR5-Δ32 heterozygosity; the frequency of CCR5-Δ32 heterozygotes was found to be disproportionately high in long-term nonprogressors from our own cohort as well as others (27, 29, 33, 34). Among seroconverters from the Multicenter AIDS Cohort Study, heterozygotes had slower rates of decline in CD4+ T cell counts and lower levels of plasma viremia 9–18 mo after seroconversion compared with CCR5 wild-type individuals (28). Although no delay in progression to AIDS could be detected among heterozygotes in that study, two other studies which combined seroconverters from several cohorts did find that CCR5-Δ32 heterozygosity correlated with a delay in progression to AIDS (27, 29). Data regarding the lower levels of plasma viremia 9–18 mo after seroconversion in CCR5-Δ32 heterozygotes (28) are compelling, given the prognostic value of this measurement (40, 41). Therefore, one plausible mechanism for nonprogression with HIV infection may be a lower viral load set-point after acute infection due to reduced availability of functional CCR5 coreceptors in CCR5-Δ32 heterozygotes.

It is clear that heterozygosity for the mutant CCR5 gene is not the sole or even dominant explanation for nonprogression in HIV disease, since CCR5 wild-type nonprogressors are also able to achieve the same high-CD4+ T cell count/low-viral load phenotype. In this regard, it is possible that in certain CCR5 wild-type individuals, downregulation of CCR5 expression in anatomical compartments critical for establishment and propagation of HIV infection (i.e., genital and oropharyngeal mucosa, lymphoid tissue) might mimic the phenotype that results from mutation in the CCR5 gene. A similar phenotype might result from limited coreceptor availability caused by elevated levels of the ligands for CCR5, namely RANTES, MIP-1 α , and MIP-1 β . Although we found no difference in the serum levels of these β -chemokines in CCR5 wild-type versus heterozygous nonprogressors, a possibility currently under investigation in our laboratory is that important differences in levels of these chemokines may exist at the microenvironmental level where viral replication occurs (i.e., lymphoid tissue).

Another possible explanation for the failure of HIV-infected CCR5-Δ32 heterozygotes to constitute a distinct subgroup of nonprogressors could involve virologic factors. Michael et al. showed that any salutary role against disease progression played by CCR5-Δ32 was negated when CCR5-Δ32 heterozygotes harbored syncytium-inducing (SI) viruses which preferentially use CXCR4 rather than CCR5 as their coreceptor (33). Thus, the results of this study could be biased if there was skewing in the distribution of SI and NSI viruses such that CCR5-Δ32 heterozygotes preferentially harbored SI viruses. However, as we and others have reported previously, the number of HIV-infected long-term nonprogressors harboring SI viruses is negligible (8, 42), arguing against any skewing in the distribution of SI and NSI viruses between CCR5 wild-type and CCR5-Δ32 heterozygous nonprogressors.

Although our data support a role for the mutant CCR5 allele in the determination of the state of nonprogression in some HIV-infected individuals, other host and viral factors are also likely to be involved. The enormously complex interactions which occur between virus and host are obviously influ-

enced by the products of the host's MHC genes. A number of epidemiologic studies have found a strong association between MHC haplotypes and rates of HIV-1 disease progression (43–46). Genetic polymorphisms in host genes that are used by the virus for its own replication, or by the host in mounting an immune response against the virus, are also likely to play key roles in determining the rate of disease progression in infected individuals. The literature is replete with such examples in infectious, autoimmune, and neoplastic diseases (47–49). In this regard, a recent report describes an effect of variant alleles of mannose-binding lectin on susceptibility to HIV infection and disease progression (50). With regard to HIV-1 coreceptors, it is possible that polymorphisms in the CCR5 gene other than the 32-bp deletion may exist and play a role in determining the rate of disease progression. The same is true for polymorphisms in the CXCR4 (fusin) gene (51), the product of which functions as a coreceptor for SI strains of HIV-1 (52, 53). Genetic and regulatory features of CXCR4 expression as well as those of its natural ligand SDF-1 (54, 55) are currently under investigation in our laboratory as potential factors involved in long-term nonprogression with HIV-1 infection. Finally, many groups, including our own, continue to seek associations be-

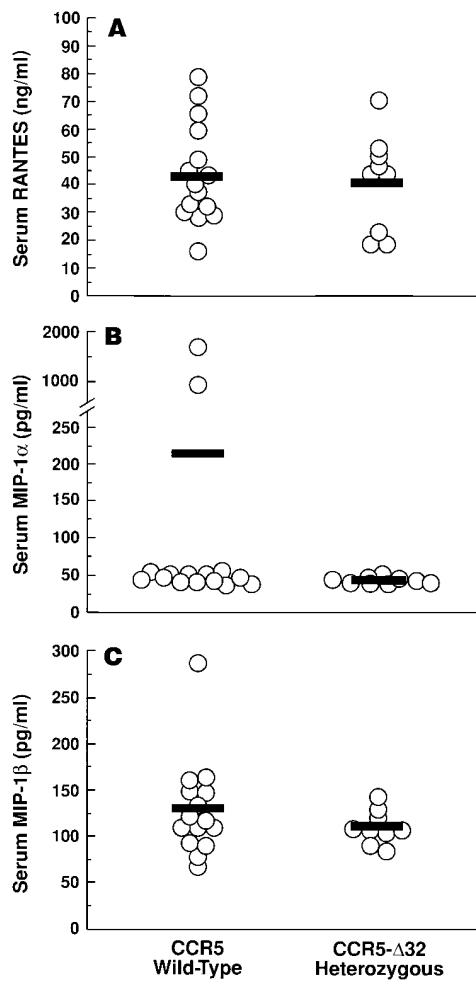


Figure 5. Comparative analysis of serum levels of the β -chemokines RANTES (A), MIP-1 α (B), and MIP-1 β (C) in CCR5 wild-type and CCR5-Δ32 heterozygous long-term nonprogressors. Mean values are indicated by horizontal bars.

tween nonprogression and various quantitative and qualitative aspects of the humoral and cell-mediated immune responses against HIV-1 (6–8, 56–59).

In summary, although a disproportionately high frequency of CCR5-Δ32 heterozygotes was found among long-term non-progressors, the hypothesis that this partial genetic defect might lead to lower viral loads and higher CD4+ T cell counts compared to CCR5 wild-type individuals could not be validated. Importantly, we measured viral load not only in the peripheral blood compartment, but also in lymphoid tissue, a critical reservoir and site of viral replication (35, 38, 60, 61). Despite our inability to distinguish CCR5 wild-type non-progressors from CCR5-Δ32 heterozygous nonprogressors based on CD4+ T cell counts and several independent measures of viral load several years after primary infection, the mutant CCR5 allele still might play a role in nonprogression in HIV infection by limiting viral replication during acute infection and thus establishing a lower set point of viremia early on in the course of infection. Finally, this study fortifies the concept that HIV-infected long-term nonprogressors indeed represent a heterogeneous group with multiple host and viral factors likely to be responsible for their common phenotype of low viral loads and preserved immune function.

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