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Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression

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ABSTRACT **RANTES (regulated on activation normal T cell expressed and secreted) is one of the natural ligands for the chemokine receptor CCR5 and potently suppresses *in vitro* replication of the R5 strains of HIV-1, which use CCR5 as a coreceptor. Previous studies showed that peripheral blood mononuclear cells or CD4$^+$ lymphocytes obtained from different individuals had wide variations in their ability to secrete RANTES. These findings prompted us to analyze the upstream noncoding region of the RANTES gene, which contains cis-acting elements involved in RANTES promoter activity, in 272 HIV-1-infected and 193 non-HIV-1-infected individuals in Japan. Our results showed that there were two polymorphic positions, one of which was associated with reduced CD4$^+$ lymphocyte depletion rates during untreated periods in HIV-1-infected individuals. This mutation, RANTES−28G, occurred at an allele frequency of ≈17% in the non-HIV-1-infected Japanese population and exerted no influence on the incidence of HIV-1 infection. Functional analyses of RANTES promoter activity indicated that the RANTES−28G mutation increases transcription of the RANTES gene. Taken together, these data suggest that the RANTES−28G mutation increases RANTES expression in HIV-1-infected individuals and thus delays the progression of the HIV-1 disease.**

The chemokine receptor CCR5 is an essential coreceptor for the cellular entry of R5 strains (macrophage tropic/non-syncytium-inducing strains) of HIV-1 (1–6), which predominate in the early stages of infection (7). During the course of infection, variants called X4 strains (T cell-line tropic/syncytium-inducing strains) emerge (1, 8–11), which use CXCR4 as a coreceptor (12). *In vitro* replication of R5 strains can be blocked by the ligands for CCR5, macrophage inflammatory peptide-1α and -1β, and RANTES (regulated on activation normal T cell expressed and secreted; refs. 13 and 14), whereas that of X4 strains can be blocked by the CXCR4 ligands stromal cell derived factor-1α and -1β (15, 16).

Mutations in HIV-1 coreceptors and their natural ligand genes have been shown to modify HIV-1 transmission and disease progression. Individuals homozygous for a 32-nt deletion in the CCR5 coding region were resistant to HIV-1 infection (17, 18), whereas heterozygosity delays disease progression (19, 20). A single V-to-I substitution in the first transmembrane segment of CCR2, a minor coreceptor for dual tropic R5X4 strains (3, 5), has a significant impact on disease progression but not on HIV-1 transmission in cohorts of seroconverters (21, 22). Finally, homozygosity of a single G-to-A mutation in the 3′ noncoding region of the stromal cell derived factor-1β gene also showed a disease-retarding effect (23), although later studies could not confirm this effect (24, 25).

Among three natural CCR5 ligands, RANTES showed the highest potency to suppress *in vitro* replication of R5 strains of HIV-1 (13). Phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) or CD4$^+$ enriched lymphocytes obtained from different individuals have shown wide variations in their ability to secrete RANTES and macrophage inflammatory peptide-1β (26, 27), and the levels of these chemokines secreted in the culture supernatants were correlated inversely with rates of HIV-1 disease progression (27, 28). Furthermore, CD4$^+$ lymphocytes from exposed but uninfected individuals secreted more RANTES than those from individuals infected with HIV-1 (26, 29–31). These pieces of evidence of differential levels of RANTES secretion among individuals prompted our search for polymorphisms in the upstream noncoding region of the RANTES gene, which contains cis-acting elements involved in RANTES promoter activity (32), in HIV-1 infected and non-HIV-1-infected individuals in Japan. We show here that there are two polymorphic positions, one of which is associated with a delay in disease progression. Functional analyses of RANTES promoter activity show that the mutation associated with a delay in disease progression increases transcription of the RANTES gene.

MATERIALS AND METHODS

Clinical Samples. Blood from 272 HIV-1-infected and 193 non-HIV-1-infected Japanese individuals was collected and treated with heparin. HIV-1-infected cases include 144 hemophiliacs, most of whom were infected through contaminated blood products between 1982 and 1985. Non-HIV-1-infected cases include 50 hemophiliacs who might have been exposed to contaminated blood products in the same period without being infected with HIV-1. PBMC were obtained from blood by using the Ficoll/Hypaque (Amersham Pharmacia) method. DNA was extracted from the PBMC by using a method previously described (33). Total RNA was extracted from the

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Abbreviations: RANTES, regulated on activation normal T cell expressed and secreted; PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cell.
Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB023652–AB023654).
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PBMC by using Trizol (GIBCO). $CD4^+$ lymphocyte depletion rates were calculated for 126 HIV-1-infected individuals who had five or more $CD4^+$ lymphocyte counts recorded during untreated periods. Of these individuals, 112 had 10 or more $CD4^+$ lymphocyte counts recorded, and 97 of them had 20 or more.

Statistical Analysis. The unpaired *t* test was used.

Genotyping of RANTES Gene. DNA fragments corresponding to a 1,031-bp upstream noncoding region of the RANTES gene were PCR-amplified by using a primer pair of RA1 (5′-AGAAGGCCTTACAGTGAGA-3′) and RA3 (5′-GCGCAGAGGGCAGTAGCAA-3′; Fig. 1). The amplified region contained 907 bp of the immediate 5′ upstream region of the major transcription start site, the 5′ (68-bp) untranslated region, and 56 bp of the 5′ coding region (Fig. 1). PCR was performed in a 50-μl reaction mixture containing 1 μg of DNA (40 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min); 386-bp cDNA fragments spanning the entire coding region of RANTES were reverse-transcribed from mRNA by using primer Rm6 (5′-GGACAAGAGCAAGCAGAAAC-3′) and PCR-amplified by using the primer pair of Rm6 and Rm1 (5′-GCAGAGGATCAAGACAGCAC-3′). PCR was performed in a 50-μl reaction mixture containing 1 μg of total RNA (30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s). Amplified DNA fragments were purified and sequenced by using primers RA3, RA2F (5′-GACCCTCCTCAATAAAAC-3′), and RA2R (5′-TGGCAGTTAGGACAGGAT-3′) for the RANTES promoter (Fig. 1) and primers Rm1 and Rm6 for the RANTES coding region. Sequencing reactions were performed according to the dideoxy-chain-termination method by using the ABI prism 377 (Applied Biosystems) automated DNA sequencer.

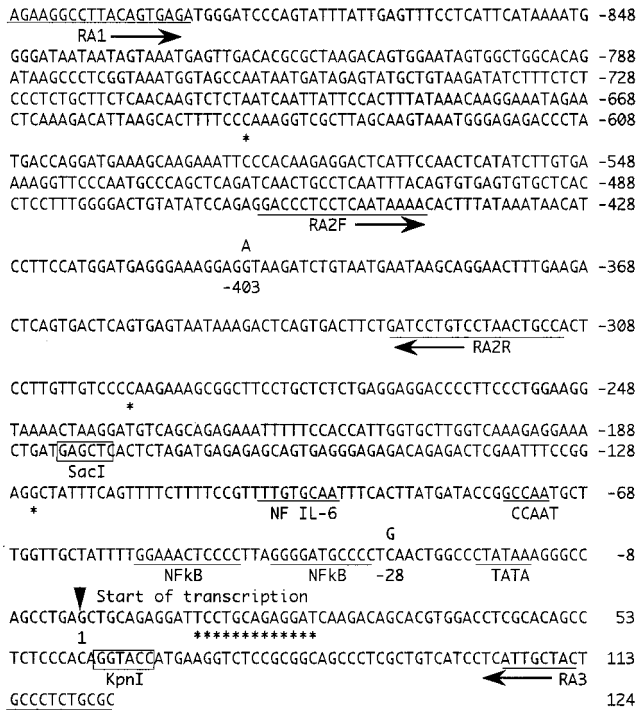

FIG. 1. Nucleotide sequence of RANTES promoter region. Numbers indicate nucleotide positions relative to the major transcription start site marked by a triangle. The numbers −403 and −28 indicate the two polymorphic positions. Possible consensus sites for NFκB, NFIL6, CCAAT, and TATA are underlined. Primer positions used for PCR amplification and sequence determination are underlined with arrows, indicating the direction of the primers. *Sac*I and *Kpn*I restriction-enzyme sites used to generate the luciferase plasmid are boxed. Asterisks below the sequence indicate the nucleotides that were not present in a previous report (32). Each of our sequences possesses these additional nucleotides. At present, it is unclear whether or not these differences represent sequence polymorphisms between white and Japanese individuals.

Quantitation of RANTES Levels. $CD4^+$ enriched lymphocytes were obtained from PBMC by depleting $CD8^+$ cells by using immunomagnetic beads (Dynal, Great Neck, NY). After activation with 5 μg/ml PHA for 7 days in the presence of 20 units/ml interleukin 2, 0.8×10^6 $CD4^+$ enriched lymphocytes were incubated in 1.5 ml of growth medium for an additional 4 days. Supernatants were assayed for RANTES levels by using the commercially available RANTES ELISA Kit (R & D Systems).

Luciferase Reporter Gene Assays. DNA fragments corresponding to the sequences spanning −907 to +68 and −182 to +68 of the RANTES promoter were inserted in the multiple cloning site of the pGL3-Basic Vector carrying the firefly luciferase gene (Promega). All constructs were verified for sequence authenticity; 5 μg of the resultant constructs was transfected with DOTAP (Boehringer Mannheim) into colon carcinoma SW480 cells or with DMRIE-C (GIBCO/BRL) into monocytic U937 cells and $CD4^+$ T lymphocytic Jurkat cells. Transfection efficiency was normalized by cotransfecting 0.2 μg of pRL-TK, which expresses *Renilla* luciferase under the control of the herpes simplex virus thymidine kinase promoter. Cells were harvested 40 h after transfection, and firefly and *Renilla* luciferase activities were determined according to manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega). Relative luciferase expression was derived with the following equation: (firefly luciferase activity of RANTES promoter construct/*Renilla* luciferase activity)/(firefly luciferase activity of promoterless vector pGL3-Basic/*Renilla* luciferase activity). Data points are means derived from measurements of two independent clones of each construct.

RESULTS

Polymorphism in RANTES Chemokine Promoter. Evidence of differential RANTES secretion levels among individuals (26–31) prompted our search for polymorphisms that may affect HIV-1 transmission and/or disease progression. We sequenced a 1,031-bp PCR-amplified fragment of the upstream noncoding region of the RANTES gene in 30 HIV-1-infected and 33 non-HIV-1-infected individuals. We identified polymorphisms at two positions: a C-to-G substitution at position −28 and a G-to-A substitution at position −403 (Fig. 1 and Table 1). Because both mutations were found in the 3′ half of the sequenced region, we sequenced a 540-bp fragment of this region in an additional 242 HIV-1-infected and 160 non-HIV-1-infected cases in Japan.

We observed six genotypes in both HIV-1-infected and non-HIV-1-infected cases (Table 1). The following genotypes were not detected: G/A at −403 and G/G at −28, G/G at −403 and C/G at −28, and G/G at −403 and G/G at −28. The fact that these genotypes were not detected suggests the absence of a haplotype containing a G at both positions −403 and −28. Therefore, at least three haplotypes of the RANTES promoter are present in the Japanese population (haplotypes I, II, and III in Table 1). The frequencies of haplotypes I, II, and III in non-HIV-1-infected subjects were 62.4%, 21.0%, and 16.6%, respectively. There was no significant difference in the frequencies of each genotype and haplotype between HIV-1-infected and non-HIV-1-infected cases (Table 1), suggesting that the mutations do not exert a protective effect against HIV-1 transmission. The distribution of these haplotypes is in Hardy–Weinberg equilibrium, indicating a lack of selection for or against each.

Haplotype III Is Associated with Reduced CD4 Depletion Rates. We examined the effect of RANTES promoter mutations on the rate of HIV-1 disease progression. Among 272

Table 1. Frequencies of RANTES promoter genotypes and haplotypes among HIV-1-infected and non-HIV-1-infected individuals

	Genotype/ haplotype		No. non-HIV-1-infected (frequency, %)			No. HIV-1-infected (frequency, %)		
	−403	−28	Total	Hemophiliacs	Nonhemophiliacs	Total	Hemophiliacs	Nonhemophiliacs
Genotype								
1	G/G	C/C	79 (40.9)	16 (32.0)	63 (44.0)	117 (43.0)	64 (44.4)	53 (41.4)
2	A/A	C/C	9 (4.7)	3 (6.0)	6 (4.2)	13 (4.8)	6 (4.2)	7 (5.5)
3	A/A	G/G	8 (4.1)	1 (2.0)	7 (4.9)	11 (4.0)	3 (2.1)	8 (6.2)
4	G/A	C/C	49 (25.4)	18 (36.0)	31 (21.7)	69 (25.4)	40 (27.8)	29 (22.7)
5	G/A	C/G	34 (17.6)	7 (14.0)	27 (18.9)	48 (17.6)	26 (18.1)	22 (17.2)
6	A/A	C/G	14 (7.3)	5 (10.0)	9 (6.3)	14 (5.2)	5 (3.5)	9 (7.0)
Total			193 (100)	50 (100)	143 (100)	272 (100)	144 (100)	128 (100)
Haplotype								
I	G	C	241 (62.4)	57 (57.0)	184 (64.3)	351 (64.5)	194 (67.4)	157 (61.3)
II	A	C	81 (21.0)	29 (29.0)	52 (18.2)	109 (20.0)	57 (19.8)	52 (20.3)
III	A	G	64 (16.6)	14 (14.0)	50 (17.5)	84 (15.5)	37 (12.8)	47 (18.4)
Total			386 (100)	100 (100)	286 (100)	544 (100)	288 (100)	256 (100)

infected subjects, we were able to calculate $CD4^+$ lymphocyte depletion rates in 126 cases (51 subjects with genotype 1, 6 with genotype 2, 5 with genotype 3, 31 with genotype 4, 27 with genotype 5, and 6 with genotype 6) during untreated periods. As shown in Fig. 2, individuals either homozygous or heterozygous for haplotype III (genotypes 3, 5, and 6) showed significantly slower rates of $CD4^+$ lymphocyte depletion than those without haplotype III (genotypes 1, 2, and 4), suggesting that haplotype III plays a role in delaying disease progression. On the other hand, haplotypes I and II showed no protective effect against disease progression (Fig. 2).

To exclude the possibility that the observed effect of haplotype III on HIV-1 disease progression was caused by linked mutations in the RANTES coding region, we determined the sequences of cDNA to RANTES mRNA spanning the entire coding region in two subjects with genotype 1, three with genotype 2, two with genotype 3, and two with genotype 6. No mutations were observed in the RANTES coding regions in these individuals.

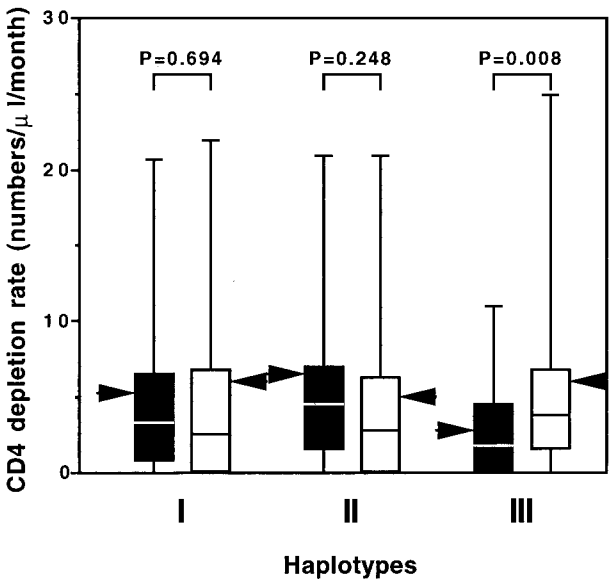

FIG. 2. Effect of sequence polymorphism in RANTES promoter region on $CD4^+$ lymphocyte depletion rate. The bars represent the 25th and 75th percentiles for the $CD4^+$ depletion rate; a horizontal line in the bar represents the 50th (median) percentile and small horizontal lines above and below the bar represent the 5th and the 95th percentiles. Homozygotes and heterozygotes of each haplotype are represented by a black bar. Those lacking each respective haplotype are represented by a white bar. The mean $CD4^+$ depletion rate of each group is shown by an arrowhead. Statistical significance for each difference is indicated.

Expression Levels of RANTES. The apparent protective effect of haplotype III against disease progression prompted us to assess serum RANTES levels in 37 non-HIV-1-infected individuals with various genotypes (15 with haplotype III and 22 without haplotype III). Serum RANTES levels varied among subjects, ranging from 11 ng/ml to 42 ng/ml. Contrary to our expectations, we did not observe a significant difference in serum RANTES levels between individuals with haplotype III and those without haplotype III (Fig. 3*A*). We then measured RANTES levels in culture supernatants of PHA-stimulated $CD4^+$ lymphocytes obtained from the same 37 individuals. RANTES levels in culture supernatants also varied greatly among subjects, ranging from 20 pg/ml to 1,300 pg/ml (Fig. 3*B*). However, we observed that $CD4^+$ lymphocytes from subjects with haplotype III secreted significantly more RANTES (mean titer = 398.0 pg/ml) than those from subjects without haplotype III (mean titer = 177.3 pg/ml; $P = 0.014$).

Functional Analysis of RANTES Promoter. The C-to-G mutation at −28 (RANTES−28G), specific for haplotype III, is adjacent to the NFκB binding site (Fig. 1). We therefore constructed luciferase reporter fusions to compare promoter activity among haplotypes I, II, and III. Constructs L1, L2, and L3 contained nucleotide sequences from −907 to +68 of haplotypes I, II, and III, respectively. Our results showed that L3 expressed slight but significantly higher luciferase activity than L1 and L2 in colon carcinoma SW480 cells, U937 monocytic cells (Fig. 4), and a $CD4^+$ Jurkat T cell line (data not shown). To test whether −28G is sufficient to increase promoter activity, we constructed an artificial RANTES promoter (L4) carrying a G at both positions −28 and −403. This construct also showed elevated luciferase activity, indicating that RANTES−28G is responsible for elevated promoter activity (Fig. 4). We additionally constructed two shorter versions of the RANTES promoter-luciferase fusions (spanning positions −182 to +68). The short construct carrying RANTES−28G (Fig. 4, S2) exhibited slightly higher luciferase activity than its nonmutant counterpart (Fig. 4, S1), confirming the critical role of RANTES−28G in elevating promoter activity.

DISCUSSION

In this report, we have shown that a single point mutation in the RANTES promoter, RANTES−28G, is associated with reduced $CD4^+$ lymphocyte depletion rates in HIV-1-infected

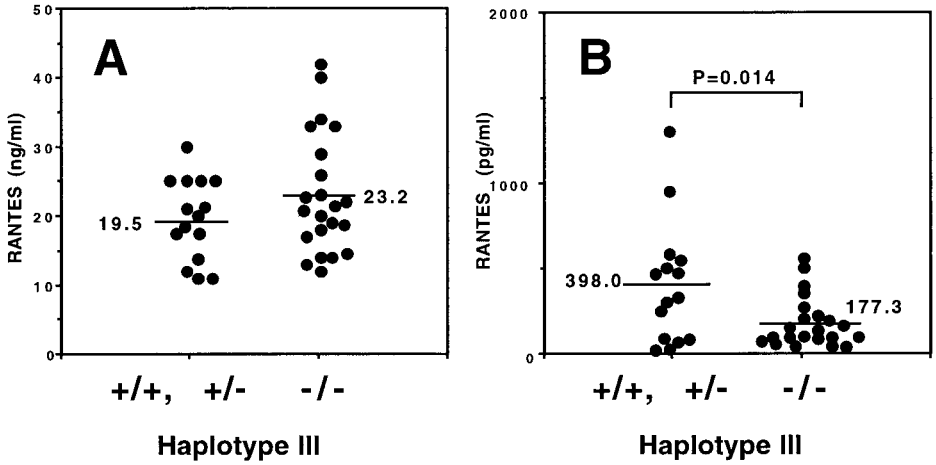

FIG. 3. RANTES secretion levels in serum (*A*) and culture supernatants of PHA-stimulated $CD4^+$ enriched lymphocytes (*B*) obtained from 37 uninfected individuals. The symbols +/+ and +/− denote homozygote and heterozygote for haplotype III, respectively, and −/− denotes those lacking haplotype III. Horizontal lines indicate the mean levels. Statistical significance for difference is indicated.

individuals. The RANTES−28G was shown to increase promoter activity of RANTES in two different luciferase reporter gene constructs, suggesting that this mutation increases RANTES expression in a human body. Therefore, it is tempting to speculate that RANTES−28G increases RANTES expression within HIV-1-infected individuals, thus hindering viral spread and disease progression. This view is highly relevant to the recent discoveries that lymphocytes from individuals with long-term nonprogressing disease or individuals who were exposed but uninfected secreted more RANTES than those from individuals with normally progressing disease after PHA-stimulation (26–31). The similarity in serum RANTES levels between non-HIV-1-infected subjects with RANTES−28G and those without RANTES−28G may explain why RANTES−28G does not affect HIV-1 transmission.

Although activated $CD4^+$ lymphocytes bearing RANTES−28G secreted significantly more RANTES than those without RANTES−28G, 5 of 15 subjects with haplotype III secreted low levels of RANTES ranging from 20 pg/ml to 85 pg/ml (Fig. 3*B*). These results suggest that RANTES−28G alone does not determine RANTES secretion levels from $CD4^+$ lymphocytes. RANTES is known to have complicated patterns of expression (34), and many factors are involved in temporal changes of RANTES expression in $CD4^+$ lymphocytes (35, 36). It is possible that polymorphisms in these factors together with RANTES−28G determine RANTES expression levels in $CD4^+$ lymphocytes. Alternatively, RANTES−28G may increase RANTES promoter activity in cell types other than $CD4^+$ lymphocytes.

At present, it is difficult to explain the reason for the complete absence of a haplotype bearing G at both positions −403 and −28. The activity of the artificial promoter bearing G at both −403 and −28 was shown to be similar to haplotype III promoter in three different cell lines. One possible explanation for the absence of this haplotype is that the G-to-A mutation in haplotype II may have evolutionarily antedated the C-to-G mutation in haplotype III. However, a possibility that the promoter bearing G at both −403 and −28 has a lethal effect in the context of the chromosome cannot be excluded at present.

In conclusion, we have identified a polymorphism in the RANTES promoter that hinders HIV-1 disease progression. Recently, a mutation in CCR2 was reported to show different effects on disease progression in whites and in African-Americans (25). Thus, it is important to analyze the RANTES promoter regions of whites and Africans, whose mutant frequencies have not yet been reported. It is also important to investigate the combined effects of mutations in CCR5, CCR2,

FIG. 4. The effect of sequence polymorphism in the RANTES promoter region on promoter activity in SW480 and U937 cells. The promoter regions inserted into the pGL3-Basic vector are shown by solid lines with the first and last nucleotides indicated. Nucleotides at the two polymorphic positions (−403 and −28) are indicated. Boxes indicate firefly luciferase ORFs. The relative luciferase activity of each construct is represented by solid bars. Data shown are representative of four independent experiments with similar results. Error bars account for fluctuations between measurements of relative luciferase activity in two independent clones of each construct.

stromal cell derived factor-1β and RANTES on HIV-1 transmission and disease progression.

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