PREVALENCE OF MUTANT CCR5 ALLELE IN SLOVENIAN HIV-1-INFECTED AND NON-INFECTED INDIVIDUALS

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Summary. – A 32 bp deletion in the CCR5 gene designated CCR5Δ32 has been identified recently as the cellular basis for resistance to human immunodeficiency virus type 1 (HIV-1) in some individuals which remained non-infected despite a repeated exposure to this virus. The prevalence of this deletion was examined by polymerase chain reaction (PCR) on 51 HIV-1-infected and 385 non-infected individuals from all parts of Slovenia. 84.4% of the the HIV-1-infected and 83.2% of the non-infected individuals were homozygous for wild type CCR5, and 19.6% and 16.3%, respectively, were heterozygous. No homozygous mutant genotype was observed among the HIV-1-infected patients. Of the non-infected individuals, 2 women (0.5%) were found to harbour the CCR5Δ32/CCR5Δ32 genotype only, which is, to the best of our knowledge, the lowest prevalence of this particular genotype found among Caucasians to date.

Key words: human immunodeficiency virus type 1; acquired immunodeficiency syndrome; CCR5 gene; chemokine receptor; Slovenia

Introduction

The β-chemokine receptor 5 (CCR5) is a major co-receptor on $CD4^+$ cells for macrophage-tropic, syncytium-non-inducing isolates of HIV-1. Rare HIV-1 strains are able to use CCR2b or CCR3 as co-receptors, while T cell line-tropic, syncytium-inducing HIV-1 isolates preferentially use fusin. Macrophage-tropic viruses are predominantly transmitted sexually and are present throughout the course of infection (Deng *et al.*, 1996; Huang *et al.*, 1996).

A 32 bp deletion in the CCR5 gene (CCR5Δ32) has been identified recently as the cellular basis for resistance to HIV-1 in some individuals which remained non-infected despite repeated exposure to HIV-1 (Liu *et al.*, 1996; Paxton *et al.*, 1996). Thus, when transfected into cells, the mutant gene produces a protein that is not expressed on the cell surface and does not support HIV-1 entry (Samson *et al.*, 1996; Broder and Collman, 1997). Several large population-based studies provided compelling evidence for high level of protection against HIV-1 infection in individuals who were homozygous for CCR5Δ32 (Huang *et al.*, 1996; Michael *et al.*, 1997). In contrast, the role of CCR5Δ32 in heterozygous individuals, i.e. individuals with one normal copy of the gene, remains inconclusive. Although some authors found fewer heterozygotes among infected than non-infected groups, a finding suggesting partial protection against HIV-1 infection (Samson *et al.*, 1996), others found no evidence for protection against infection but rather a limited protection against the disease progression in heterozygous individuals (Huang *et al.*, 1996; Michael *et al.*, 1997).

Approximately 1% of randomly selected HIV-1-negative Caucasian individuals in Western Europe and the United States and up to 30% of highly exposed non-infected individuals had homozygous CCR5Δ32 genotype. Up to 16% of individuals in these populations were heterozygotes for CCR5Δ32. In contrast, CCR5Δ32 was infrequent among African Americans and absent in black Africans, Japanese, and Venezuelan populations studied to date (Liu *et al.*, 1996; Samson *et al.*, 1996; Broder and Collman, 1997; Huang *et al.*, 1997).

Abbreviations: AIDS = acquired immunodeficiency syndrome; HIV = human immunodeficiency virus; PCR = polymerase chain reaction

The prevalence of CCR5Δ32 among HIV-1-infected and non-infected individuals in the South-eastern Europe, especially among Slavs, to the best of our knowledge, has not been clarified yet. In order to elucidate this question, we have conducted a molecular epidemiological study on 51 HIV-1-infected and 385 non-infected individuals from all parts of Slovenia.

Material and Methods

Selection of samples. The first group consisted of 51 HIV-1 seropositive individuals that had attended the Department of Infectious Diseases at the Clinical Centre of Ljubljana (the only hospital in Slovenia in which HIV-infected persons are followed up and treated) between January and August 1997. This group included 38 males and 13 females of age range of 17 – 63 years (the mean 36.6 years). According to the Center for Diseases Control classification (C.D.C., 1992) the patients belonged to the following groups: A1 (n=4), A2 (n=20), A3 (n=4), B2 (n=2), B3 (n=3), C1 (n=1) and C3 (n=17). The risk factors for HIV-1 infection were homosexuality/bisexuality in 28 cases, haemophilia in 8 cases, heterosexual intercourse with an HIV-1-infected subject in 8 cases, intravenous drug use in 4 cases, intravenous drug use in combination with prostitution in 2 cases, and bite of HIV-1-infected person in terminal stage of infection in 1 case (Vidmar *et al.*, 1996). At the time of the study, 6 patients had below 2,000 copies of HIV-1 RNA/ml, 15 patients 2,000 – 15,000 copies, 8 patients 15,000 – 50,000 copies, 7 patients 50,000 – 100,000 copies, and 15 patients up to 100.000 copies as determined by the Amplicor HIV-1 Monitor Assay (Roche Molecular Systems, Branchburg, NJ, USA).

For the group of HIV-1 non-infected individuals, 385 samples were randomly retrieved from 743 frozen lymphocyte samples stored in the Slovenian AIDS Reference Centre. These samples were obtained from the same Slovenian volunteers who had participated in previous Slovenian HIV-1 prevalence surveys. All individuals included in the second group claimed never to have injected drugs and had no history of blood transfusion, surgical operation or invasive procedures, and did not travel recently to countries with a high prevalence of HIV infection. This group included 217 males and 168 females of age range of 16 – 65 years (the mean 36.2 years). All randomly selected individuals were clearly negative for the presence of anti-HIV-1 and anti-HIV-2 antibodies in serum samples using two different screening enzyme immunoassays (ICE HIV-1.0.2 Test, Murex Diagnostics, Dartford, Great Britain, and Enzygnost Anti-HIV 1+2 Test, Behring AG, Marburg, Germany). The anti-HIV tests were performed according to the manufacturer's directions (Poljak *et al.*, 1997).

Detection of CCR5Δ32. Duplicate whole blood samples were collected from each of 436 individuals tested in the study. Within 3 hrs of collection, two to five 0.5 ml aliquots of whole blood from each individual's sample were each processed into cell pellets using the Whole Blood Specimen Preparation Kit (Roche Diagnostic Systems, Branchburg, NJ, USA) according to the manufacturer's directions (Poljak *et al.*, 1996). The cell pellets kept at -70°C before amplification were incubated with 200 µl of the DNA extraction reagent at 60°C for 30 mins and then at 98°C for 30 mins. Two µl of extracted DNA sample was added to 98 µl of a reaction mixture containing 200 µmol/l each of dATP, dCTP, dGTP, and 500 µmol/l dUTP, 10 mmol/l Tris.HCl pH 8.3, 50 mmol/l KCl, 3 mmol/l $MgCl_2$, 0.01% gelatin, 50 pmoles of the primers CCR5c (5'-CAAAAAGAAGGTCTTCATTACACC-3') and CCR5d (5'-CCTGTGCCTCTTCTTCTCATTTCG-3'), 0.1 U of uracil-N-glycosylase (Perkin-Elmer, Norwalk, CT, USA) and 2.5 U of Taq DNA polymerase (Boehringer Mannheim, Germany). The reaction mixture was subjected to 40 amplification cycles using a DNA Thermal Cycler 9600 (Perkin-Elmer, Norwalk, CT, USA). Each cycle consisted of 95°C for 30 secs, 60°C for 30 secs, and 72°C for 45 secs. Prior to amplification, the reaction mixture was kept at 50°C for 10 mins (activation of uracil-N-glycosylase). The final elongation cycle at 72°C extended by 10 mins. The amplification products were separated by 4% agarose gel electrophoresis (Metaphor Agarose, FMC Bio Products, Rockland, MA, USA) in TBE buffer and were visualised by UV illumination following ethidium bromide staining.

All known precautions to avoid a PCR product carry-over and sample-to-sample contamination were rigorously taken. The different steps of the PCR procedure were performed in separate rooms using different pipettes with aerosol-resistant tips. The uracil-N-glycosylase procedure was used for prevention of false positive results due to an amplicon carry-over.

Interpretation of results. The primers CCR5c and CCR5d that flank the 32 bp deletion of CCR5 gene were used to generate wild type and deleted fragments of 189 bp and 157 bp, respectively. In case that only the 189 bp fragment was obtained, the result was interpreted as the wild type homozygous genotype (CCR5/CCR5). The presence of the 157 bp fragment only was interpreted as the 32 bp deletion homozygous genotype (CCR5Δ32/ CCR5Δ32), and the presence of both the fragments as the heterozygous genotype (CCR5Δ32/CCR5). Representative examples of each genotype are presented in Fig. 1.

Statistical analysis of results was performed by the χ^2 test.

Results and Discussion

In the present study, two groups of individuals (51 HIV-1-infected and 385 non-infected from all parts of Slovenia were tested for the presence of CCR5Δ32 deletion using PCR. The obtained results are summarised in Table 1. A higher frequency of homozygous CCR5/CCR5 genotype was found among non-infected individuals and a higher prevalence of heterozygous CCR5Δ32/CCR5 genotype among HIV-1-infected individuals. Although the prevalences of both CCR5/CCR5 and CCR5Δ32/CCR5 genotypes obtained in our study were similar to those found in other Caucasians populations (Huang *et al.*,1996; Liu *et al.*, 1996; Samson *et al.*, 1996; Michael *et al.*, 1997), in contrast to these studies, the differences between the prevalences among Slovenian HIV-1-infected and non-infected individuals were found statistically insignificant (Table 1). In agreement with previous studies (Huang *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996; Michael *et al.*, 1997), no homozygous

Table 1. CCR5 genotype frequencies among HIV-1-infected and non-infected individuals from Slovenia

CCR5 genotype	HIV-1 infected (n=51)	HIV-1 non-infected (n=385)	P
CCR5/CCR5	41 (80.4%)	320 (83 2%)	>0.05
CCRΔ32/CCR5	10 (19.6%)	63 (16.3%)	>0.05
CCR5Δ32/CCR5Δ32	0 (0.0%)	2 (0.5%)	>0.05

CCR5Δ32/CCR5Δ32 genotype was observed among Slovenian HIV-1-infected patients. In Slovenian non-infected individuals, only 2 women (0.5%) were found to harbour homozygous CCR5Δ32/CCR5Δ32 genotype. The chi-square analysis indicated that the prevalence of the homozygous CCR5Δ32/CCR5Δ32 genotype in non-infected Slovenians was significantly lower as compared to other Caucasian populations studied to date (in all comparisons $P < 0.05$ and χ^2 ranged from 4.1 to 7.6). The biological significance of the lower prevalence of homozygous CCR5Δ32/CCR5Δ32 genotype among Slavs (or at least Slovenians) in comparison with other Caucasians remains to be determined. It is of interest that no pathology has been associated with CCR5Δ32/CCR5Δ32 homozygosity indicating that CCR5 is at present not essential to human health. It is not clear why the CCR5 gene is dispensable in humans, but this phenomenon may reflect environmental factors (Moore *et al.*, 1997). One of possible explanations may be that CCR5 is important for protection of humans against some microorganisms endemic only in some parts of the world and is not essential in other parts of the world.

In conclusion, we found in the present study the lowest prevalence of homozygous CCR5Δ32/CCR5Δ32 genotype among Caucasians to date. We think that similar studies on other Slavic populations are indispensable to prove whether the low prevalence of mutant homozygous CCR5 genotype found in our study is characteristic only for Slovenians or according to common origin of all Slavs for all of them.

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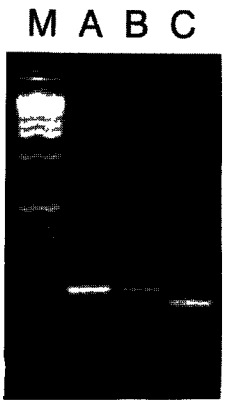

Fig. 1
Agarose gel electrophoresis of PCR products from DNAs from Slovenian individuals
A: CCR5/CCR5 genotype (189 bp band); B: CCR5Δ32/CCR5 genotype (189 bp and 157 bp bands); C: CCR5Δ32/CCR5Δ32 genotype (157 bp band); M: 1 kb molecular mass ladder.

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