Abstract. The frequency of CCR5-Δ32 allele in human immunodeficiency virus type 1 (HIV-1) infection in the southern Brazilian population was determined in a cross-sectional study carried out from October 2001 to June 2004. Genomic DNA was extracted from peripheral blood cells of 134 healthy blood donors, 145 HIV-1-exposed seronegative individuals, 152 HIV-1-seropositive asymptomatic individuals, and 478 HIV-1-seropositive individuals with AIDS. A fragment with 225 base-pairs of the CCR5 gene was amplified by polymerase chain reaction. The CCR5-Δ32 homozygous deletion was observed in 2 (1.5%) blood donors and in 1 (0.7%) individual HIV-1-exposed seronegative, and was absent among all the HIV-1-seropositive individuals (Fisher’s exact test, p=0.0242). The frequency of the homozygous CCR5-Δ32 deletion in the HIV-1-exposed did not differ when compared with that observed in the HIV-1 seronegative blood donors (Fisher’s exact test, p=0.6093; OR: 2.18, 95% CI: 0.11-129.6). The wild-type genotype CCR5/CCR5 frequency was higher among the HIV-1-seropositive with AIDS compared to HIV-1 seropositive asymptomatic individuals (Chi-square test, p=0.0263; OR: 2.02, 95% CI: 1.03-3.97). The absence of the homozygous deletion of CCR5-Δ32 among HIV-1-seropositive individuals underscored that this genotype is an important genetic factor associated with the decreased susceptibility to HIV-1 infection. The higher frequency of heterozygosity for the CCR5-Δ32 and the CCR5-Δ32 allele in HIV-1 seropositive asymptomatic compared to HIV-seropositive with AIDS individuals also underscored that this deletion could be associated with the delay of the HIV-1 disease progression in this population. However, the low frequency of CCR5-Δ32 homozygosity observed among HIV-1-exposed seronegative individuals shows that the allele could not explain, by itself, the natural resistance to HIV-1 infection and different mechanisms of protection against HIV-1 infection that must be involved in this population.

Introduction

The natural history and pathogenic processes of infection by the human immunodeficiency virus type 1 (HIV-1) are complex, variable, and the ability to control the infection and to delay the progression to AIDS and/or death is probably regulated by a balance between host and viral factors (1-5). Human allelic variants influence not only the susceptibility to HIV-1 infection but also the subsequent rates of disease progression towards AIDS. The deletion of 32-base-pairs in the chemokine receptor 5 gene (CCR5-Δ32) was the first and most well characterized host restriction allele associated with a high degree of protection against HIV-1 infection (6). Homozygous individuals for CCR5-Δ32 allele have been found to be resistant to HIV-1 infection, while the heterozygous can significantly delay the onset of AIDS. This deletion creates a shortened protein which remains intracellular and fails to reach the cell surface in homozygous individuals for this variant. Although the heterozygosity was not related to
the complete protection against HIV-1 infection (6,8), it may confer partial protection against disease progression or death in HIV-1 infected individuals (9-16). Heterozygosity for the CCR5-Δ32 deletion is significantly higher in cohorts of HIV-1 infected long-term non-progressors (LTNP) compared to HIV-1 infected typical progressor individuals (9,17,18).

The global, ethnic and regional distribution of the CCR5-Δ32 allele varies significantly giving each population a different genetic resistance profile to the HIV-1 infection and AIDS progression (19). The CCR5-Δ32 allele is relatively common in Caucasians whose allelic frequency is ~10%, and homozygosity is found in 1% of Caucasian blood donors (20). The CCR5-Δ32 deletion is prevalent in European populations with wide variation of 10-20% and almost absent from Asian groups (8). The frequency of this deletion was observed with a gradient, uppermost at the north around the Baltic Sea down to the Mediterranean coast (21). In European-derived populations from the US and Southern Europe, CCR5-Δ32 was observed at a frequency of 5-10%, but decreased to 2-5% in Hispanic populations, throughout the Middle East or in the Indian continent. This allele is almost absent among African-Americans in whom admixture with people of European descent has been considerable (20). In Asian populations, the CCR5-Δ32 allele was absent among Japanese, Filipino, Korean, Chinese and Indian populations studied (22), and healthy Thai blood donors (23). No Δ32/Δ32 genotype was detected among normal individuals from south of Iran (24), and from Arabic countries such as Egyptians and Syrians (25).

In some countries of Latin America, the frequency of the CCR5-Δ32 allele was 5.3% and absent among individuals from Venezuela or in Amerindian (20,26). In Brazil, studies of the CCR5 genotypes carried out in urban unrelated healthy individuals found 93.0% of wild-type CCR5/CCR5 homozygotes, 7.0% of CCR5/Δ2 heterozygotes, and absence of homozygous Δ2/Δ2 individuals, resulting in an allelic frequency of 0.035 (27). Further, the absence of CCR5-Δ32 allele was also observed in Amerindians from four Brazilian Amazon tribes. All the individuals were homozygous for the wild-type allele, which correlates to the hypothesis that the CCR5-Δ32 allele has an European origin, and that its occurrence in urban populations in South America is the result of immigration (28). Among HIV-1 seronegative and HIV-1 infected individuals from northeast Brazilian region, the CCR5-Δ32 allelic frequency was 0.026 and 0.044, respectively; among German-descended healthy blood donors from southern region was 0.065; and was absent among individuals of two Amerindian tribes from north region (29). Another study carried out in unrelated healthy individuals from southern Brazilian region, no homozygotes for the CCR5-Δ32 deletion were detected (30).

The HIV-1 epidemic is increasing in Brazil, where over 620,000 (370,000 to one million) people living with HIV-1 were identified (31). Up to June 2006, a total of 433,067 cases of AIDS had been reported in Brazil, and the southern state of Paraná has the fifth highest incidence of AIDS in the country, with 20,176 cases (32). However, previous studies involving the CCR5-Δ32 allele and HIV-1 infection evaluated a small number of individuals. The present study was carried out in order to determine the frequency of CCR5-Δ32 allele in a large Brazilian sample of healthy blood donors, HIV-1-exposed seronegative individuals, and HIV-1-seropositive individuals in various stages of HIV-1 infection.

Materials and methods

Participants and design. The protocol was approved by the Institutional Research Ethics Committees of Londrina State University and of the Health State Secretariat of Paraná, in southern Brazil. Individuals were invited to participate and informed in detail about the research, and their written consent was obtained. The sample evaluated was available from previous studies carried out from October 2001 to June 2004 and were described previously (33-35). For the CCR5 genotype study, a cross-sectional study was carried out and 909 individuals were enrolled. All of them were classified into self-described racial characteristics and distributed into four groups:

Blood donors. One hundred and thirty-four healthy HIV-1 seronegative individuals recruited from faithful and repeatedly screened blood donors of the Blood Bank of the University Hospital of Londrina, Paraná state, with a number of previous blood donations ranging from 2-17 (4.4±2.6). They were considered to be at low risk of HIV-1 infection according to the risk-screening strategies for determining individuals at risk for HIV-1 infection (36).

HIV-1-exposed but seronegative. One hundred and forty-five HIV-1-exposed individuals but seronegative after ≥2 consecutive anti-HIV-1 antibody tests by immunological assays. The individual risk was determined through risk screening based on self-report behavioral risks (36). All the 145 individuals had high exposure to HIV-1 infection through repeatedly unprotected sexual intercourse with a known HIV-1-infected partner for at least 2 years prior to the present study. Fifty individuals also reported repeated exposure (≥3 episodes) to blood infected with HIV-1 infection by sharing equipment for injectable drug use (needles, syringes, cotton, water) with HIV-1-seropositive individuals. All participants were advised on safe-sex practice.

HIV-1-seropositive asymptomatic. One hundred and fifty-two HIV-1-seropositive individuals, asymptomatic and with CD4+ T-cell counts ≥350 cells/mm³. The most frequent risk factor for HIV-1 infection was sexual contact (n=142). Other risk factors also reported were blood contact by sharing equipment for injectable drug use (needles, syringes, cotton, water) with HIV-1-seropositive individuals or blood transfusion, and accident with blood (n=20). Some individuals reported more than one risk factor for HIV-1 infection. At the time of enrollment, 98 (64.5%) subjects were receiving no antiretroviral therapy, and 54 (35.5%) were on antiretroviral therapy, according to the Brazilian guidelines that were used at that time (37).

HIV-1 seropositive with AIDS. Four hundred and seventy-eight HIV-1-seropositive individuals with the symptoms of the disease and/or CD4+ T-cell counts <350 cells/mm³, according to the Brazilian government criteria definition (38). The most
frequent risk factor for HIV-1 infection was sexual contact (n=420); blood contact by sharing equipment for injectable drug use (needles, syringes, cotton, water) with HIV-1-seropositive individuals, blood transfusion, accident with blood, vertical transmission, and tattoo (n=89). Some individuals also reported more than one risk factor for HIV-1 infection. At the time of enrollment, 104 (21.7%) subjects were receiving no antiretroviral therapy, according to the Brazilian guidelines that were used at that time (37).

The HIV-1-exposed seronegative and the HIV-1-seropositive individuals were enrolled in several specialized, public, and nonprofit centers for diagnosis and treatment of STD, including University Hospital of Londrina State University; Outpatient Clinic Hospital of Londrina State University; Integrated Center of Infectious Diseases of Londrina, and the Health Services of several cities from Paraná state, southern Brazil. These individuals presented relative homogeneity in demographic characteristics such as gender, age, risk factors for HIV-1 infection, route of transmission of the HIV-1, access to free health care and relatively uniform clinical, diagnostic procedures, and treatment patterns for the HIV-1 infection, as described previously (33-35).

### Table I. Frequency (%) of CCR5-Δ32 deletion according to the race of the HIV-1-seronegative and HIV-1-seropositive individuals from a southern Brazilian population.

<table>
<thead>
<tr>
<th>CCR5 genotypesa</th>
<th>Caucasians (n=635)</th>
<th>Mullatoes (n=214)</th>
<th>Blacks (n=55)</th>
<th>Asians (n=5)</th>
<th>Total (n=909)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5/CCR5</td>
<td>574 (90.4)</td>
<td>202 (94.4)</td>
<td>55 (100.0)</td>
<td>5 (100.0)</td>
<td>836 (92.0)</td>
</tr>
<tr>
<td>CCR5/Δ32</td>
<td>58 (9.1)</td>
<td>12 (5.6)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>70 (7.7)</td>
</tr>
<tr>
<td>Δ32/Δ32</td>
<td>3 (0.5)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>3 (0.3)</td>
</tr>
<tr>
<td>CCR5/Δ32 allele frequency</td>
<td>0.0504</td>
<td>0.0280</td>
<td>0</td>
<td>0</td>
<td>0.0418</td>
</tr>
</tbody>
</table>

aCCR5/CCR5, wild-type genotype; CCR5/Δ32, heterozygous genotype for the deletion; Δ32/Δ32, homozygous genotype for the deletion. Fisher's exact test, p=0.077.

Serological, and immunological assays. The serological screening tests used were enzyme immunosorbent assay (ELISA, Murex™ HIV-1.2.0, Murex Biotech Limited, Dartford, UK), and microparticle enzyme-immunoassay (MEIA, Abbott Axsym™ System, HIV-1/2 gO, Abbott GmbH, Weisbaden, Germany). The confirmatory tests used were indirect immunofluorescence (slides from Fiocruz Institute, Rio de Janeiro, Brazil) and Western blot analysis (Genelabs Diagnostics, Singapore). Both screening and confirmatory tests were performed according to the Brazilian government standard procedures (39). The first determination of CD4+ and CD8+ T-cell counts, obtained after the date of enrollment, was performed for 909 individuals by flow cytofluorimetry (FACSCount™, Becton, Dickinson and Company, San Jose, CA) and the results were recorded as number of cells/mm³.

CCR5 genotyping. The CCR5 genotypes were determined by polymerase chain reaction (PCR) as previously described (6,13). Briefly, a peripheral blood sample was collected, with EDTA as anticoagulant. Mononuclear cells were separated by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient. Genomic DNA was extracted using the salting out method described elsewhere (40). The following primers were used from the previously published sequences (GenBank accession no.: AF 009962): primer sense: 5’-ACCAGATCTCTAAAA GAA-3’ and primer anti-sense: 5’-CATGATGGTGAAG TAAAGCTCA-3’. For amplification, a total volume of 25 μl, containing 2.0 μl (100 ng) of genomic DNA, 1.5 μl (2.5 μM) of each primers, 2.0 μl (1,25 μM) of dNTPs mix (Invitrogen™ Life Technologies, Carlsbad, CA), 0.75 μl MgCl₂ (50 μM), 2.5 μl 10 X PCR buffer (500 mM KCl and 200 mM Tris-HCl, pH 8.4), 2.5 μl (2 units) of Taq DNA polymerase (Invitrogen Life Technologies), 12.25 μl of sterile water, and 2.0 were used. PCR was performed for 35 cycles (Hybaid Sprint™ thermocycler, Biosystems, Barcelona, Spain), each consisting of denaturation at 94˚C for 1 min, annealing at 58˚C for 1 min and extension at 72˚C for 1 min. Pre-denaturation and further extension were performed at 94˚C for 5 min and 72˚C for 10 min, respectively. The PCR products of 225 base-pair fragment from wild-type allele and of 193 base-pair fragment from the deleted allele were analysed by 3% agarose gel electrophoresis and visualized by UV fluorescence after staining with ethidium bromide. Ambiguous results were resolved by performing electrophoresis of the PCR products in a 10% acrylamide gel, and the gel stained with silver. In each experiment reaction, both control amplification containing no added DNA and positive control containing known CCR5 wild-type genotype were included. The images of the gel were captured and recorded with the Digit-Doc-It Program version 1.1.25.

Statistical analysis. A database with the results was set up using the EPI INFO software version 6.04d (41) and SAS Program (42). Comparisons between race were performed using the Fisher’s exact test. In this study, two main hypotheses were tested. The first was that homozygosity for the CCR5-Δ32 deletion would be higher among HIV-1-exposed seronegative individuals compared to HIV-1 seronegative blood donors. The second was that the wild-type genotype (CCR5/CCR5) frequency would be higher in HIV-1 seropositive with AIDS compared to HIV-1-seropositive asymptomatic individuals.
The odds ratio (OR) with 95% confidence interval (CI), and Fisher’s exact test was used to test the first hypothesis and Chi-square test was used to test the second hypothesis. Two-tailed tests were used, and differences between groups were considered to be statistically significant at p<0.05.

**Results**

**Frequency of CCR5-Δ32 allele according to the race of HIV-1-seronegative and HIV-1-seropositive individuals.** In a total of 909 samples evaluated, the CCR5 genotype polymorphism distribution and CCR5-Δ32 allelic frequencies were not different when the self-reported racial characteristic of the individuals evaluated was considered (Fisher’s exact test, p=0.077). The allelic frequency was 0.0504 among the Caucasian individuals and the CCR5-Δ32 allele was absent among the Black and Asian individuals (Table I).

**Frequency of CCR5-Δ32 in HIV-1-seronegative blood donors, HIV-1-exposed seronegative and HIV-1-seropositive individuals.** The frequencies of the CCR5-Δ32 deletion were 0.0559 in healthy blood donors, 0.0551 in HIV-1-exposed seronegative, 0.0559 in HIV-1 seropositive asymptomatic and 0.0292 in HIV-1 seropositivity with AIDS (Fisher’s exact test, p=0.0242) (Table II). Eleven (8.2%) out of 134 HIV-1-seronegative blood donors were heterozygous and two (1.5%) were homozygous for the CCR5-Δ32 deletion. Fourteen (9.6%) out of 145 HIV-1-exposed seronegative were heterozygous for CCR5-Δ32 and one (0.7%) was homozygous for the deletion. The frequency of the homozygous CCR5-Δ32 deletion in HIV-1-exposed did not differ when compared with that observed in HIV-1 seronegative blood donors (Fisher’s exact test, p=0.6093; OR: 2.18, 95% CI: 0.11-129.6) when the frequency of CCR5-Δ32 homozygous mutation observed in HIV-1-exposed seronegative was compared with the observed in HIV-1-seronegative blood donors; Fisher’s exact test, p=0.0287, when the frequency of CCR5-Δ32 allele observed in all HIV-1-seronegative individuals was compared with that observed in all HIV-1-seropositive individuals; Chi-square test, p=0.0263 (OR: 2.02, 95% CI: 1.03-3.97) when the frequency of wild-type genotype observed in HIV-1 seropositive with AIDS was compared with that observed in HIV-1-seropositive asymptomatic individuals.

**Comparison of the frequency of CCR5-Δ32 in HIV-1-seropositive asymptomatic individuals versus HIV-1-seropositive individuals with AIDS.** Of the 152 HIV-1-seropositive asymptomatic individuals that also presented CD4+ T-cell count ≥350 cells/mm³, 17 (11.2%) were heterozygous for the deletion and 135 (88.8%) had the wild-type genotype. Of the 478 HIV-1-seropositive with AIDS and/or presented CD4+ T-cell count <350 cells/mm³, 28 (5.9%) were heterozygous for the deletion and 450 (94.1%) had the wild-type genotype (Table II). The wild-type genotype CCR5/ CCR5 frequency was higher among HIV-1-seropositive with AIDS compared to HIV-1 seropositive asymptomatic individuals (Chi-square test, p=0.0263; OR: 2.02, 95% CI: 1.03-3.97).

**Discussion**

This is the largest study undertaken to determine the frequency of the CCR5-Δ32 allele in a Brazilian population of healthy individuals, HIV-1-exposed seronegative individuals, HIV-1-seropositive asymptomatic and symptomatic individuals. The overall frequencies of the homozygous genotype of the CCR5-Δ32 deletion and the CCR5-Δ32 allele obtained either for the total sample or for only the blood donors were consistent with data reported for other healthy populations from Latin America and Europe (6-8,10,21,26,27,29,43).

The overall allelic frequency of 0.418 obtained can be explained by the heterogeneous ethnic structure of the Brazilian population. Europeans, Asians, Africans and native Amerindians that contributed to the formation of the present Brazilian population since the 16th century.

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Table II. Distribution of the CCR5 genetic polymorphism and frequency of CCR5-Δ32 deletion in HIV-1-seronegative and HIV-1-seropositive individuals from a southern Brazilian population.

<table>
<thead>
<tr>
<th>CCR5 genotype</th>
<th>HIV-1 seronegative</th>
<th>HIV-1 seropositive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood donors</td>
<td>HIV-1-exposed</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td></td>
<td>(n=134)</td>
<td>(n=145)</td>
</tr>
<tr>
<td>CCR5/CCR5</td>
<td>121 (90.3)</td>
<td>130 (89.7)</td>
</tr>
<tr>
<td>CCR5/Δ32</td>
<td>11 (8.2)</td>
<td>14 (9.6)</td>
</tr>
<tr>
<td>Δ32/Δ32</td>
<td>2 (1.5)</td>
<td>1 (0.7)</td>
</tr>
<tr>
<td>CCR5-Δ32 allele</td>
<td>0.0559</td>
<td>0.0551</td>
</tr>
</tbody>
</table>

*aCCR5/CCR5, wild-type genotype; CCR5/Δ32, heterozygous genotype for the deletion; Δ32/Δ32: homozygous genotype for the deletion. bHIV-1-exposed but uninfected sexual partners of HIV-1-infected individuals. cHIV-1-seropositive asymptomatic individuals and with CD4+ T-cell count ≥350/mm³. dHIV-1-seropositive individuals with the symptoms of the disease and/or CD4+ T-cell count <350/mm³. Fisher’s exact test, p=0.0263 (OR: 2.02, 95% CI: 1.03-3.97) when the frequency of CCR5-Δ32 allele observed in all HIV-1-seronegative individuals was compared with that observed in all HIV-1-seropositive individuals; Fisher’s exact test, p=0.0287, when the frequency of CCR5-Δ32 allele observed in all HIV-1-seropositive individuals was compared with that observed in all HIV-1-seropositive individuals; Chi-square test, p=0.0263 (OR: 2.02, 95% CI: 1.03-3.97) when the frequency of wild-type genotype observed in HIV-1 seropositive with AIDS was compared with that observed in HIV-1-seropositive asymptomatic individuals.
(44,45). The absence of the CCR5-Δ32 homozygous genotype observed in Black and Asian people was in agreement with previous studies showing this genotype is infrequent among populations of African or Asian origins (8,20,22,23).

The similar frequency of the CCR5-Δ32 allele observed among healthy individuals and HIV-1-exposed seronegative individuals evaluated in this study, despite their different risk factors for HIV-1 infection, is consistent with the hypothesis that this allele could not explain, by itself, the natural resistance to HIV-1 infection among sexually exposed to HIV-1 but seronegative individuals. This fact underscores the existence of different mechanisms responsible for natural resistance to HIV-1 infection as suggested by other studies (22,26,42,46,47).

Host factors modulating viral entry such as genetic polymorphisms in other chemokine receptors or in their ligands (48), the innate and acquired cellular immune responses against HIV-1 (3,49), and HLA genes (50-52) have been suggested to be involved both in the resistance to HIV-1 infection and disease progression. Several groups that are at risk for HIV-1 infection but have not become infected have been reported and studies have shown that some of these persons demonstrate HIV-1-specific cytotoxic T lymphocyte (CTLs) response as well, suggesting a possible role of CTLs in preventing establishment of infection (53-59).

The absence of the homozygous for the CCR5-Δ32 deletion among HIV-1-seropositive individuals is in agreement with previous studies that showed this host as an important genetic factor associated with the decreased susceptibility to HIV-1 infection (6,7). The higher frequency of heterozygosity for the CCR5-Δ32 observed in HIV-1-seropositive asymptomatic individuals than in HIV-1 seropositive with AIDS is also consistent with studies carried out in other worldwide populations. The heterozygosity was significantly higher in cohorts of HIV-1 infected LTNP compared to HIV-1 infected typical progressors (9,17,18). Although the heterozygosity was not related to the complete protection against HIV-1 infection (6,8), it may confer partial protection against disease progression or death in HIV-1 infected individuals (9,12,14,15). Presumably, heterozygosity limits the number of coreceptors available for HIV-1 binding. CCR5 density of the surface of the CD4+ T-cell has been correlated with viral load in persons with untreated HIV-1 infection (60). Studies incorporating viral phenotype have suggested that the protective effect of CCR5-Δ32 heterozygosity against disease progression is lost when the infection virus is T-tropic (61).

An international meta-analysis showed that HIV-1 infected subjects heterozygous for the CCR5-Δ32 displayed lower HIV-1 RNA level than wild-type patients. This result appears to be supported by the simple explanation that fewer available CCR5 portals on cells of CCR5-Δ32 delay HIV-1 replication and the virus-mediated destruction of the CD4+CCR5+ T-cell lymphocyte population (16).

The relative homogeneity of the sample evaluated may contribute to the validation of the results obtained and to strengthen the earlier observations that the effect of CCR5-Δ22 mutation, by itself, may not be sufficient to prevent the risk of acquisition of the HIV-1 infection but is related to the delayed disease progression. In this post-genomic era, the observation that the naturally-occurring genetic CCR5-Δ32 mutation has been associated with delayed disease progression underscores the importance of host factors in the clinical course of HIV-1 infection, addresses fundamental issues in our understanding of the susceptibility, resistance and pathogenesis of several diseases and consolidates the importance of therapeutic intervention with combinations of chemokine receptor inhibitors to improve the antiretroviral therapeutic effects.

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