

1. Fang G, Kuiken C, Weiser B, Rowland-Jones S, Plummer F, Chen CH, Kaul R, Anzala AO, Bwayo J, Kimani J, Philpott SM, Kitchen C, Sinsheimer JS, Gaschen B, Lang D, Shi B, Kemal KS, Rostron T, Brunner C, Beddows S, Sattenau Q, Paxinos E, **Oyugi J**, Burger H. Long-term survivors in Nairobi: complete HIV-1 RNA sequences and immunogenetic associations. *J Infect Dis.* 2004 Aug 15; 190 (4):697-701.

## Abstract

To investigate African long-term survivors (LTSs) infected with non-subtype B human immunodeficiency virus type 1 (HIV-1), we obtained full-length HIV-1 RNA sequences and immunogenetic profiles from 6 untreated women enrolled in the Pumwani Sex Worker Cohort in Nairobi, Kenya. There were no discernible sequence changes likely to cause attenuation. CCR2-V64I, an immunogenetic polymorphism linked to LTSs, was detected in 4 women, all of whom carried the HLA B58 allele. Further investigation of 99 HIV-1-infected Nairobi women found an association between CCR2-V64I and HLA B58 ( $P = .0048$ ). Studying the interaction among immunogenetics, immune responses, and viral sequences from all HIV-1 subtypes may increase our understanding of slow HIV-1 disease progression.

Sub-Saharan Africa accounts for 70% of HIV-1-infected individuals globally, and infected women in this region outnumber men. The study of long-term survivors (LTSs) is relevant to pathogenesis and the design of an HIV-1 vaccine. The HIV-1 subtypes and host immunogenetics of LTSs in Africa differ from those of most LTSs studied previously [6-6]; for example, the HLA types are more diverse [6], and  $\Delta 32$  mutations in coreceptor CCR5 are rarely seen [4].

Recombination between different HIV-1 subtypes has been well documented [2, 3, 7] and, along with viral diversity, is also relevant to the design of a vaccine. Analysis of both viral diversity and intersubtype recombination would benefit from the sequencing of entire viral genomes derived from plasma virions. The examination of plasma HIV-1 RNA offers an opportunity to observe the replicating virus population, including recombinant genomes in circulating viral particles. Nairobi sex workers, who are exposed to a range of viral strains, may be infected with intersubtype recombinants. To investigate pathogenesis in women with non-clade B HIV-1 infection, we analyzed complete HIV-1 RNA sequences, immunogenetic traits, immune responses, coreceptor utilization, and drug resistance in untreated LTSs from Kenya.

**Subjects and methods.** The subjects were untreated HIV-1-infected adult women enrolled in the Pumwani Sex Worker Cohort in Nairobi, Kenya [1]. The research was approved by the Kenyatta National Hospital National Ethical and Scientific Review Committee, the University of Manitoba Use of Human Subjects in Research Committee, and the New York State Department of Health Institutional Review Board.

Within this cohort, long-term nonprogressors (LTNPs) and LTSs were identified [1]. Both LTSs and LTNPs had been infected with HIV-1 for  $\geq 10$  years, and LTNPs had maintained CD4<sup>+</sup> T cell counts  $\geq 500$  cells/ $\mu$ L. To detect coreceptor polymorphisms, human genotyping was performed as

described elsewhere [4, 8]. Extraction of viral RNA from plasma, reverse transcription, long polymerase chain reaction (PCR) amplification, and analysis of full-length HIV-1 sequences were performed as described elsewhere [7]. Phylogenetic trees were constructed, and HIV-1 subtypes and recombinants were determined as described elsewhere [7]. Full-length HIV-1 *env* genes were cloned from plasma, and coreceptor usage was determined phenotypically by the use of GHOST cells [9]. The V3 loop sequence of *env* clones was also determined and was used to genotypically predict coreceptor utilization [10]. Molecular class I HLA types were determined as described elsewhere [6]. Neutralizing antibodies were detected as described elsewhere [11]. Genotypic resistance to antiretroviral agents was analyzed by the ADRA program [2]; phenotypic resistance was measured by the PhenoSense assay [12].

Several nonparametric tests of association were used to correlate the immunogenetic data with the virologic and clinical data. A 2-sided Wilcoxon rank sum test was used to test differences between continuous measures such as the number of CD4<sup>+</sup> T cells and viral load. Fisher's exact test was used to analyze HLA types and coreceptor polymorphisms.

A number of genetic analyses were performed by use of the Mendel statistical package [13]. Hardy-Weinberg-equilibrium (HWE) testing was used to examine whether the genotype frequencies for single loci were equal to products of the population allele frequencies. Gamete-phase equilibrium testing, a generalization of linkage-equilibrium testing that allows one to consider loci on different chromosomes, would normally be used to examine whether the joint frequencies of different alleles at several loci derived from the same parent are the product of the underlying population allele frequencies. To test gamete-phase equilibrium (or linkage equilibrium), however, knowledge of the parental source of the alleles at a locus (i.e., phase information) is required. Because phase information was unavailable but multilocus genotypes were known, we instead tested for genetic equilibrium. Genetic equilibrium holds only when both HWE and gamete-phase equilibrium are maintained. If genetic equilibrium was violated but separate tests of HWE were not rejected at all loci, then we assumed that genetic disequilibrium was a result of gamete-phase disequilibrium.

**Results.** [Table 1](#) shows the clinical, virologic, immunologic, and immunogenetic characteristics of 6 subjects in the Nairobi female sex-worker cohort who had been infected with nonsubtype B HIV-1 for  $\geq 10$  years. Virions were isolated from plasma obtained from all 6 subjects in 1997 and also from additional plasma obtained in 1986 from subject ML013. The complete RNA genome was reverse-transcribed, amplified by long PCR, and directly sequenced.

[View larger version:](#)

[In this page](#)

[In a new window](#)

[Download as PowerPoint Slide](#)

### Table 1.

Virologic and immunogenetic characteristics in 6 untreated women in the Pumwani Sex Worker Cohort in Nairobi, Kenya.

Complete HIV-1 RNA sequences were assembled, aligned, and analyzed by computational methods [7]. GenBank accession numbers are shown in [table 1](#). Three subjects—ML752, ML013, and ML605—were infected with HIV-1 genomes identified as entirely subtype A; both the 1986 and the 1997 samples from ML013 also displayed subtype A genomes. Subject ML415 was infected with a viral genome identified as entirely subtype D. Two subjects displayed HIV-1 genomes that were intersubtype recombinants. Virus from ML672 was composed predominantly of clade A sequences with a clade C fragment in the pol gene. Subject ML249's recombinant virus was predominantly composed of clade D but also displayed a clade C fragment in nef and the 3' long terminal repeat.

Sequences were examined for mutations that might contribute to attenuation of HIV-1. It is possible that single-nucleotide changes might help to attenuate the virus, and it was reported recently that R77Q, a mutation in the HIV-1 *vpr* gene, is associated with both LTNP infection and impaired induction of apoptosis [14]. This mutation was present in 3 of the 6 women studied, including 2 of the LTNPs ([table 1](#)); the association, however, was not statistically significant. No other clearly attenuating mutations or deletions were detected, nor any polymorphisms common to more than 1 sequence.

We determined human genotypes for HIV-1 coreceptors, coreceptor-associated genes, and HLA class I haplotypes, to examine the contribution of immunogenetics to LTSs ([table 1](#)). All 6 subjects had homozygous wild-type CCR5 genotypes.

Four subjects exhibited polymorphisms in the CCR2 gene; 2 LTNPs (ML672 and ML752) were homozygous for the V64I mutation, and 2 LTS subjects (ML013 and ML605) were heterozygous for it. It is noteworthy that all 4 women who carried the V64I allele also displayed the B58 HLA haplotype. Statistical analyses showed an association between the presence of the CCR2-V64I mutation (in at least 1 allele) and HLA type B58 ( $P=.06$ ).

To explore this association further, we expanded our immunogenetic analysis to include a larger group of 167 women in the Nairobi sex-worker cohort [1]. In addition to CCR2 and HLA B58, we examined the SDF-1  $\alpha$ -3' untranslated region, bringing the total to 3 human genes, each located on a different chromosome [4]. There was no significant association between the CCR2 mutation and the SDF mutation. As shown in [table 2](#), 99 (59.3%) of the 167 women were HIV-1 seropositive and 68 (40.7%) were HIV-1 seronegative. The B58 allele was of interest; all other alleles were combined, and the locus was treated as biallelic. No significant deviations from HWE were found for either CCR2 or HLA-B, either in the entire sample of 167 women or in the groups

stratified by HIV serostatus. In contrast, we did find, in the entire sample, significant evidence for gamete-phase disequilibrium between CCR2 and HLA B ( $P=.00780$ ), indicating a highly significant association between CCR2-V64I and HLA type B58. This association was also significant in the HIV-seropositive subjects ( $P=.00486$ ), but not in the HIV-seronegative subjects.

|               | HIV-negative subjects <sup>a</sup> |            | HIV-positive subjects <sup>b</sup> |            | All subjects <sup>c</sup> |            |
|---------------|------------------------------------|------------|------------------------------------|------------|---------------------------|------------|
|               | HLA B58*                           | HLA B58*   | HLA B58*                           | HLA B58*   | HLA B58*                  | HLA B58*   |
| CCR2 genotype | 32 (81.5%)                         | 6 (15.6%)  | 52 (89.3%)                         | 7 (12.1%)  | 84 (88.4%)                | 13 (13.6%) |
| Viral subtype | 18 (44.6%)                         | 10 (25.6%) | 17 (29.3%)                         | 14 (23.8%) | 35 (37.3%)                | 24 (25.0%) |
| V64I/V64I     | 2 (5.0%)                           | 0 (0%)     | 6 (10.5%)                          | 2 (3.4%)   | 8 (8.5%)                  | 2 (2.1%)   |

NOTE: Data are no. (%) of women.  
<sup>a</sup>  $P < .001$ , Fisher's exact test.  
<sup>b</sup>  $P = .002$ .  
<sup>c</sup>  $P = .004$ .

View larger version:

[In this page](#)

[In a new window](#)

[Download as PowerPoint Slide](#)

## Table 2.

CCR2 mutations and HLA B58 in 167 Kenyan women in the Pumwani Sex Worker Cohort in Nairobi, Kenya.

We determined CCR5-promoter genotypes ([table 1](#)). One LTNP (ML415) and one LTS (ML249) were homozygous for CCR5-59029G, a polymorphism associated with delayed progression of HIV-1 disease [4].

Coreceptor usage was determined for HIV-1 envelope clones obtained from 5 subjects ([table 1](#)). The majority (71/77 [92.2%]) of clones utilized CCR5. A minority of CXCR4-utilizing species were also detected in 3 subjects (ML672, ML752, and ML605).

No significant drug-resistance mutations were seen. The Pheno-Sense assay was used to examine phenotypic resistance; only a specimen from subject ML415 gave a result, and no resistance was found.

Although the absence of viable cells precluded functional studies of CTL activity, we were able to predict, on the basis of the donor HLA haplotype and predicted epitopes found in the immunology databases in the Los Alamos National Laboratory and Oxford University, the likely sites of CTL recognition. At least half the predicted epitopes carried 1 or more amino acid changes from the consensus sequence (data not shown); however, KAFSPEVIPMF, the immunodominant target of CTL recognition through HLA-B57 and B58 in HIV-1 gag, was conserved in all donors [15].

Neutralizing antibody titers ranged from negative to 1:640 ([table 1](#)). The serum demonstrated neutralization against strain MN (clade B) but not against strain 92/UG/31 (clade A).

**Discussion.** This study is one of the first to characterize female LTSs and LTNPs from Africa, where both HIV-1 subtypes and immunogenetic traits differ from those of LTSs studied previously. One of the remarkable features of these LTSs is their fairly high viral loads ([table 1](#)).

These Kenyan subjects managed to survive, most of them as asymptomatics, for periods of 12–16 years, without antiretroviral treatment.

Computational analyses of the complete HIV-1 RNA sequences confirmed both the frequency of intersubtype recombination and the particular HIV-1 subtypes observed in a recent study from Kenya [3]. One LTNP (ML672) and 1 LTS (ML249) had recombinant genomes. The sequence data, which are derived from plasma virions, provide direct evidence of recombinant genomes in circulating viral particles.

Computational analyses of the sequences did not reveal any clearly attenuating mutations except for the *vpr* R77Q mutation (table 1) [14]; in our small study, the association between this mutation and LTSs was suggestive but not significant. All of the sequences analyzed in this study appeared to be intact and gave no indication that they coded for nonfunctional proteins. In fact, when multiple viral *env* genes from these subjects were cloned into an expression system to determine coreceptor utilization, most clones yielded functional envelopes. Although it is possible that 1 or more point mutations in the viral genomes may have diminished the pathogenicity of the viruses infecting these 6 women, we did not identify any deletions or mutations that would clearly confer attenuation on any of the viruses.

Immunogenetics may have contributed to LTS status in this study (table 1). CCR2-V64I, previously linked to LTSs, was detected in 4 women. A highly significant correlation between the presence of the CCR2-V64I mutation and the HLA B58 allele was found in 167 women in the Nairobi cohort. The association was particularly strong in the 99 women who were HIV-1 seropositive, although it was not significant in the 68 women who were HIV-1 seronegative. The stronger association between these 2 alleles in the infected women, compared with that in the uninfected women, may reflect, in this group, a selection for LTSs bearing both V64I and B58 genes. An alternative explanation, however, is possible, reflecting the genetics of the population under study: when 2 loci are close to each other on a chromosome, departure from gamete-phase equilibrium is often taken as evidence for linkage disequilibrium; because CCR2 and HLA B genes are on different chromosomes, departure from genetic equilibrium is likely due to recent ethnic admixture in these subjects; however, joint selective pressures may also be acting on the 2 genes.

The association of these human genes, CCR2 and HLA B58, has not been previously noted, and it may possibly provide a clue to the manner in which CCR2 affects the pace of HIV-1 infection. Although multiple studies, including 1 focusing on the Nairobi cohort [5], have reported that the CCR2-V64I allele may slow the progression of HIV-1 disease, the mechanism by which the mutation acts is still unclear [4, 5]. The HLA B57 allele, which is related to B58, has also been associated with both slowed progression of disease and long-term survival [6, 15]. The close association, in HIV-1-infected women, between the CCR2 mutation and B58 suggests that the V64I allele may affect the pace of HIV-1 infection in part or entirely through the HLA B58 haplotype. This question necessitates further investigation. Finally, these studies suggest that

studying the interaction among immunogenetics, immune responses, and viral sequences from all HIV-1 subtypes may increase our understanding of the slow progression of HIV-1 disease.