Isolation of Human Immunodeficiency Virus-Type 1 (HIV-1) clones with biological and molecular properties of the primary isolate

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Abstract

We developed a new biological cloning system for HIV-1 isolates using the U87.CD4 cell lines that express different chemokine receptors. We demonstrate that our method is sensitive and specific because the clones isolated had the same coreceptor usage and genotype as viruses of the primary isolate. We evaluated our cloning system by isolating 27 biological clones from two primary HIV-1 R3R5X4 isolates. Three HIV-1 phenotypes (R3R5X4, R3R5 and R5) were identified in isolate 29 and two (R3R5X4 or R5X4) in isolate 31. Each phenotype was distinguished by a unique genotype. Sequencing of 20 molecular clones from each isolate did not reveal additional genotypes. One of the three genotypes identified from isolate 29 was not found by molecular cloning of the original isolate, suggesting high specificity and sensitivity of the biological cloning system in isolating minor virus populations. Our results suggest that the new cloning approach can be used as an alternative to the existing method for isolating biological clones in PBMC.

Introduction

Human immunodeficiency virus type 1 (HIV-1) infects cells of the T-cell and macrophage lineages (Dalgleish et al., 1984; Folks et al., 1986; Gartner et al., 1986; Klatzmann et al., 1984). At the cell surface, the viral envelope (Env) glycoprotein (gp) 120 interacts with CD4 and a chemokine coreceptor. This interaction results in a series of conformational changes in both the gp120 and gp41 which allows the virus to enter the cell (Berger, 1997). HIV-1 has been defined as being fast or slow replicating in peripheral blood mononuclear cells (PBMC) (Asjo et al., 1986; Fenyo et al., 1988) or as having the capacity to induce syncytia in PBMC or MT-2 cells (called syncytium inducing, SI and non-syncytium inducing, NSI, respectively) (Tersmette et al., 1988). The molecular basis for the phenotypic differences of HIV-1 is due to the ability of the virus to use different coreceptors. Nowadays, it is common that HIV-1 isolates are classified as belonging to different phenotypes by their ability to use different chemokine receptors as coreceptors (Berger et al., 1998; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Feng et al., 1996). Early in infection, HIV-1 most often uses CCR5 as coreceptor and these viruses are called R5 viruses. Later in the infection, coreceptor usage of viruses may change to CXCR4 and these viruses are termed X4 viruses. The viruses may also broaden their coreceptor repertoire into using a combination of CCR5, CXCR4 and other minor chemokine receptors, mostly CCR3. In infected individuals, a switch of coreceptor usage is often associated with an accelerated loss of $CD4^+$ T-cells and progression towards clinical symptoms characteristic of acquired immunodeficiency syndrome (AIDS) (Bjorndal et al., 1997; Connor et al., 1997; Scarlatti et al., 1997; Yu et al., 1998).

The viral population of an infected individual consists of a swarm of genetically related viruses which are termed quasispecies. Quasispecies likely arise by at least two mechanisms; first, the viral replication machinery is error prone because the viral reverse transcriptase lacks proof reading ability, and second, there is continuous rapid virus turn over. While extensive genetic analyses have been carried out, less is known about the impact of quasispecies on the biological variability of HIV-1. Early work showed that SI (CXCR4-using) isolates are composed of both SI and NSI phenotypic variants (Schuitemaker et al., 1992). In agreement with these observations, we have previously shown that viruses with different coreceptor usage can be selected by limited passage in single coreceptor expressing cells (Bjorndal et al., 1997; Scarlatti et al., 1997). Coreceptor evolution plays an important role in HIV-1 pathogenesis and there is a clear correlation between appearance of CXCR4 usage and progression towards AIDS (Bjorndal et al., 1997; Karlsson et al., 1994; Koot et al., 1993).

In-depth studies on biological clones, rather than primary isolates, are important and may reveal features of the pathogenic process. It is common practice to isolate HIV-1 biological clones using PHA stimulated peripheral blood mononuclear cells (PBMC) (Schuitemaker et al., 1992). Although PBMC express the major coreceptors of HIV-1, they are present at unequal densities on the cell-surface. CXCR4 is expressed earlier after stimulation and at higher levels than CCR5, whereas CCR3 is only expressed at low levels (Bleul et al., 1997). This uneven coreceptor density may lead to a positive selection for X4 viruses at the expense of viruses with other coreceptor preference in PBMC.

To avoid selection due to uncontrolled coreceptor availability, we have developed an alternative method for isolating biological clones using the U87.CD4-system. Isolation of biological clones is performed by dilutions of viral isolates in the presence of only one coreceptor at a time.

Results presented here, using two multitropic R3R5X4 primary HIV-1 isolates, show that our method accurately identifies viral clones having the same biological and molecular properties

as the primary isolates. In addition, our approach is faster and less labor intensive than conventional cloning in PBMC.

Results

Cloning strategy and biological properties of the isolates

In comparison to PBMC, syncytia formation is easily detected in U87 cells. Cultures showing 5 or less syncytia per well were defined as having low degree of syncytia formation (LDSF). As is described below, the LDSF definition allowed us to successfully identify biological clones without determining classical "end-points" that is common practice in PBMC based cloning. Two HIV-1 primary isolates (29 and 31) were first diluted and passaged in four parallel wells on U87.CD4 cells expressing either CCR3, CCR5 or CXCR4 coreceptors. Cultures were analyzed for syncytia formation and RT activity at day 6 (for CCR5 and CXCR4 expressing cells) or at day 10 (for CCR3 expressing cells).

After first passage, the isolates reached LDSF at different dilutions on the different cell lines (Fig 2 and Fig 3). Isolate 29 scored highest both by RT and syncytia formation on CCR5-expressing cells (Fig 2) whereas isolate 31 showed highest titer on CXCR4-expressing cells (Fig 3). Isolate 29 infected CCR3-expressing cells to about the same extent as CXCR4-expressing cells (Fig 2), whereas isolate 31 infected CCR3-expressing cells at comparatively lower dilutions (Fig 3).

Isolation of biological clones

Virus supernatants from the first passage were subjected to a second selection step by infection of the three U87.CD4 coreceptor expressing cell lines. Proviral DNA was obtained from cells in the second selection step. A 300-bp region containing the V3 region of *env* was amplified and genotypes were determined by direct sequencing (Fig 1). Direct sequencing of isolate 29 showed many heterogenic sites (Fig 4A). The majority of sequences obtained after the second selection were homogenous (for example, 29A in Fig 4B and 29B in Fig 4C) whereas a few were mixtures of genetically distinct variants (for example, clone $29X_2$ in Fig 4D).

We observed a correlation between dilution of isolate 29 in the first passage and homogenous nucleotide sequence after second selection (exceptions were clones $29E_{1-3}$, see below). Isolate 29 passaged (diluted 3^3-fold) on CCR3-expressing cells had heterogeneous genotype after the second selection step (Table 1, 29 X_{1-3}). However, when LDSF was reached in the first passage (diluted both 3^5- and 3^6-fold) we observed homogenous genotypes (Table 1, clones $29A_{1-3}$ and $29B_{1-2}$, respectively). For clarity, only the V3 region is shown but clonality was observed over the entire 300-bp region sequenced. The same pattern was observed when viruses were selected on CCR5-expressing cells. In this case, the first passage of isolate 29 (a 3^3-fold dilution) on CCR5-expressing cells resulted in a mixed genotype (Table 1, $29X_{4-6}$), whereas virus that reached LDSF in first passage (diluted $4x3^5$-fold) showed homogenous genotypes (Table 1, clones $29C_{1-3}$). The genotype of $29C_{1-3}$ seemed to depend on the cells used in the second selection. Clones $29C_1$ and $29C_3$ were harvested from CCR3 and CXCR4-expressing cells, respectively, and both had genotype I, whereas clone $29C_2$ which was isolated from CCR5 expressing cells had genotype II. This observation suggested that the virus population, although highly diluted in the first step, was still heterogeneous and could be further selected in the second step. All clones obtained from CXCR4-expressing cells had a homogenous genotype (Table 1 $29E_{1-3}$ and $29F_{1-3}$) that was independent of dilution factor.

The genotype of isolate 31 was less complex compared to isolate 29 and displayed only two heterogeneous amino acid sites (Table 2). Both alternative sites were identified in the biological clones (genotype IV and V, respectively) obtained with the selective cloning

system described here. As described above for isolate 29, the efficiency for obtaining biological clones increased with the dilution factor in the first passage (Table 2).

Phenotypes of the biological clones

We next determined the phenotypes of the biological clones which we isolated using the U87-cloning system. Virus supernatants from the second selection were used to prepare viral stocks in PBMC. The phenotypes of the biological clones were determined by infecting the coreceptor expressing U87.CD4 cells with virus supernatant derived from the viral stocks. We found that isolate 29 was composed of viruses with an R3R5X4 or R3R5 phenotype, with the majority of clones being R3R5X4 (Table 1). Isolate 31 appeared to have R3R5X4 and an R5X4 population (Table 2). Interestingly, the R3R5X4 phenotype was only obtained when the first passage of isolate 31 was performed on CCR3 expressing cells. Sequence analysis showed that the charge of the V3 region differed among the biological clones. All clones from isolate 31 had a charge of +5 (Table 2) and clones from isolate 29 had a charge of +4 (R3R5) or +6 (R3R5X4) (Table 1). Pure R5 populations were not identified from any of the isolates by the method described here. This observation was surprising since isolate 29 reached highest titers on CCR5-expressing cells. We therefore subjected both isolates to serial passages on CCR5-expressing cells using diluted virus at each passage.

Serial dilutions on CCR5-expressing cells

Three consecutive dilutions were done on CCR5-expressing cells. In each passage, LDSF was reached and supernatant from the serial passages was subjected to a second selection step on CCR3, CCR5 and CXCR4 expressing cells (Fig 1). By this approach we isolated three R5 clones and one R3R5 clone (with weak CCR3 usage) from isolate 29 (Table 3, clones $29_{a\text{-}d}$). All four clones had identical sequence (genotype III). Genotype III displayed the triplet AAA (data not shown) corresponding to a lysine (K) in amino acid position 18 (Table 3), whereas genotypes I and II had the triplet AGG and AGA (data not shown), respectively, both coding for an arginine (R) (Table 1 and 2). Amplification and direct sequencing of DNA from isolate 29 identified a heterogenic site (the triplet AGG or AGA) in position 18. Thus, the AAA triplet of genotype III was not identified by direct sequencing which suggested that genotype III represented a minor fraction of the total (Leitner et al., 1993). The charge of the V3 region of genotype III was +4.

Serial dilution and passages on CCR5-expressing cells of isolate 31 confirmed the R5X4 phenotype of genotype V but we did not identify clones with pure R5 phenotype (data not shown).

Confirmation of genotypes

To confirm that the genotypes identified from the biological clones existed in the original isolates, we amplified the same *env* region as before from DNA obtained from isolates 29 and 31. Sequencing of 20 clones from each isolate confirmed all genotypes which were identified from the biological clones. The only exception was genotype III of isolate 29 (see above), again suggesting that this genotype represented a minor population. In addition, we did not identify additional genotypes in the original isolate compared to those isolated by biological cloning.

Discussion

We have developed an alternative method for isolating biological clones from primary HIV-1 isolates by using U87.CD4 cells expressing coreceptors. Biological clones are commonly isolated by limiting dilutions on human PBMC (Schuitemaker et al., 1992). Briefly, primary isolates or infected PBMC are cocultivated in 96-well plates with PHA-stimulated PBMC from healthy blood donors. The proportion (F) of infected cells is determined from the formula for the Poisson distribution, $F = -\ln(F_0)$, where F_0 is the fraction of negative cultures. Only virus clones obtained from a dilution that give rise to progeny virus in fewer than 33% of parallel cultures are considered clonal. The data presented here, suggests that our cloning system can be used as an alternative method to the existing PBMC method. The main difference between the two methods is the cells used for selection. U87 is a human glioma cell line that has been engineered to express CD4 and either CCR3, CCR5 or CXCR4. PBMC also express these coreceptors but at different levels. CXCR4 is more highly expressed than CCR5 and CCR3 is only expressed at low levels. Therefore, passage of virus isolates on PBMC will select for variants that use CXCR4 and against variants that use CCR5 and particularly those using CCR3, for example, R3R5 variants. Thus, virus variants present in small amounts and not able to use CXCR4 as a coreceptor will statistically be time and resource consuming to isolate. Our method isolated variants with only one coreceptor present at a time. In this way, the biological properties of each virus variant are taken into account and the probability to isolate variants other than CXCR4-using viruses will be higher.

Another advantage of the U87.CD4-system is that syncytia are easily detected after infection with HIV. Our results showed a correlation between isolation of biological clones, RT-activity and LDSF in the first passage. In addition, when LDSF was reached in the first passage, proviral DNA had homogenous sequences after the second selection step. Conversely, when LDSF was not reached in first passage, the identified sequences after the second selection were mixtures of more than one genotype. Therefore, LDSF alone is a good indicator of clonality and RT activity or other costly screening methods do not have to be used after the first passage. Thus, our protocol allows for fast and non-expensive selection of HIV-1 clones.

We evaluated our system using two multitropic R3R5X4 primary HIV-1 isolates (29 and 31). We obtained 18 clones from isolate 29 and 9 clones from isolate 31. The two isolates differed in their efficiency of infecting the different coreceptor expressing cell lines (Fig 2 and 3). Isolate 31 reached highest titers on CXCR4-expressing cells while isolate 29 titrated to highest levels on CCR5-expressing cells. Viruses of isolate 29 infected CCR3-expressing cells more efficiently than viruses of isolate 31. Clones isolated from the two isolates reflected these differences. Isolate 29 had clones with R5, R3R5 or R3R5X4 phenotypes, whereas clones from isolate 31 were of R5X4 or R3R5X4 phenotypes (Table 4).

Direct sequencing of amplification products from the two isolates revealed 6 and 2 heterogenic amino acids sites in isolate 29 and 31, respectively, in the V3 region (Table 1 and 2, respectively). The efficiency of our method in generating biological clones is illustrated by the fact that the heterogeneous (mixed) genotypes of the isolates were separated into different biological clones, each having a homogenous sequence that accounted for the heterogeneous sites we observed in the original isolate (Fig 4). We also observed that the efficiency in generating biological clones increased with virus dilution in first passage (Table 1 and 2). However, we were able to isolate biological clones (29_{E1-3}) when low dilution (3 fold) of isolate 29 was used in first passage on CXCR4-expressing cells. This is not surprising since our cloning system takes biological properties into account and CXCR4-using viruses were only represented by R3R5X4 viruses in isolate 29. In other words, the R3R5X4 virus population did not have to compete with other X4 strains for the CXCR4 receptor on the cells

used for first passage and lower dilutions of isolate 29 were not required to isolate biological clones on CXCR4.

Sequence analysis of the biological clones revealed 3 different genotypes in isolate 29 (genotypes I-III) and two in isolate 31 (genotypes IV and V). We found a correlation between the number of charged amino acids of the V3 loop and phenotype. CXCR4-using clones of isolate 29 had high V3 charge (+6) and a basic amino acid in position 25, whereas clones with a lower V3 charge used other coreceptors (Table 4). This observation is in agreement with several previous studies suggesting that a high V3 loop charge (de Jong et al., 1992; Fouchier et al., 1992) and a basic amino acid in position 25 (De Jong et al., 1992; Fouchier et al., 1995; Shioda et al., 1992) are associated with CXCR4 usage.

To confirm that the biological cloning system indeed selected variants preexisting in the isolate, we molecularly cloned and sequenced 20 clones from each isolate. In isolate 31, we identified all genotypes by cloning. Clones from isolate 29 were represented by genotypes I and II, but not genotype III. Since clones with genotype III (R5 phenotype) were derived through serial passage in CCR5-expressing cells, the question may arise whether this genotype is a result of mutations acquired during passages. This is highly unlikely since analysis of regions adjacent to the V3 loop from clones with genotype III, revealed several other positions that differed from sequences of other clones (data not shown). Generation of these mutations would most likely have required longer time and far more passages than we have performed. Most probably, genotype III represents a minor population (none of the 20 clones sequenced had this genotype which roughly correspond to <5% of the isolate) which confirms the selective power of our cloning system.

In this work, we have focused on analyzing clones with respect to coreceptor usage. Another important biological property is the sensitivity to neutralization. Broad resistance to neutralizing antibodies is typically lost when viruses are adapted to growth in permanent T-cell lines (Sawyer et al., 1994; Zhang et al., 1997). Moreover, a recent study showed that this is also true when viruses are passaged in PBMC (Beaumont et al., 2004). The decreased resistance to neutralizing antibodies observed may be due to the acquisition of a more open conformation of gp120 during passage (Poignard et al., 2001). This suggests that limiting the number of passages in vitro is of greater importance than the choice of cells when isolating biological clones from primary HIV-1 isolates.

The data presented here demonstrate that our new method for isolating biological clones is sensitive and specific because the clones isolated had the same coreceptor usage and genotype as viruses of the primary isolate. We also found that our method is fast and non-labor intensive and suggest that the method can be used as an alternative to the existing method for isolating biological clones in PBMC cultures.

Materials and Methods

Patients and virus isolates

Virus isolates 29 and 31 (Asjo et al., 1986; Fenyo et al., 1988) were obtained from patients with progressive HIV-1 infection and were passaged in PBMC not more than twice. The isolates have previously been shown to use CCR3, CCR5 and CXCR4 as coreceptors and were designated as R3R5X4 multitropic isolates (Bjorndal et al., 1997).

Viral stocks

Viral stocks were produced in phytohemagglutinin P (PHA-P) (Becton Dickinson and Co; USA) stimulated human PBMC. Briefly, 10^6 cells in 15 ml tubes (TPP; Switzerland) were infected with 2 ml or 900 µl of virus supernatant obtained after the second selection step. The cell/virus-mixtures were incubated at 37°C and 5% CO_2 for two hours followed by addition of 2 ml of RPMI medium (Gibco, Paisley United Kingdom) supplemented with 10% fetal bovine serum (FBS; Hyclone, Argentina), 2µg/ml polybrene (PB; Sigma, Germany), 5U of recombinant interleukein-2 (IL-2; Amersham Biosciences, Uppsala, Sweden) and penicillin/streptomycin (PEST; Gibco, Paisley, United Kingdom). The day after infection, the cells were washed with 4 ml of phosphate buffered saline (PBS; Gibco, Paisley United Kingdom) and resuspended in 10 ml of fresh RPMI medium and transferred to 25 cm^2 (TPP; Switzerland) culture flasks. The cultures were incubated at 37°C and 5% CO_2 for 7 days before virus was harvested.

Cell lines

U87.CD4 cells stably transfected with CCR3, CCR5, or CXCR4 were kindly provided by Dr. Dan Littman (Deng et al., 1996). Cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco, Paisley, United Kingdom) supplemented with 10% FBS and antibiotics. Cultures were grown in 25cm^2 tissue culture flasks and split at a ratio of 1:2 twice weekly by treatment with 5mM EDTA.

First passage

U87.CD4 cells expressing CCR3, CCR5 or CXCR4 were seeded at a density of 10^5 cells/well in 24-well plates (TPP; Switzerland) one day prior to infection. For infection of CCR3 and CXCR4 expressing cells, virus isolates were diluted in six 3-fold dilution steps, beginning with a 3-fold dilution. For CCR5-expressing cells, the dilution steps were 3^3, 3^4, 2x 3^4, 4x 3^4, 8x 3^4 and 4x 3^5-fold. Four parallel wells were infected with 400 µl of each dilution. Cells were washed with PBS and 2 ml of fresh medium was added day 1. At day 6 for CCR5 and CXCR4 expressing cells or at day 10 for CCR3-expresssing cells, post infection cultures were observed for syncytia formation and 50 µl of supernatants were used for reverse transcriptase (RT) assay (Cavidi Tech AB, Uppsala, Sweden). Remaining culture supernatants were stored at –70°C for future experiments (Fig 1).

In addition, we performed three consecutive dilutions with isolate 29 and 31 on U87.CD4-CCR5 expressing cells.

Second selection

In the second selection, 5x10^4 U87.CD4 cells expressing CCR3, CCR5 or CXCR4 were seeded in 48-well plates (TPP; Switzerland) one day prior to infection. At the day of infection,

200 µl of supernatant from first passage were used to infect the three U87.CD4-cell lines. Prior to infection supernatants from the first passage were treated with 300 Units DNase /ml (Invitrogen, Carlsbad, California, USA) for 30 minutes at room temperature. At day 1, the cells were washed with 1ml of PBS and 1 ml of fresh medium was added. Supernatants were harvested at day 6 (CCR5 and CXCR4 expressing cells) or at day 10 (CCR3-expressing cells) and used for growing viral stocks in PBMC (Albert et al., 1990) and to prepare DNA (Fig 1).

Determination of coreceptor usage of biological clones

Virus supernatants, containing 5 ng of p24 from viral stocks produced in PBMC were used to infect U87.CD4 cells expressing CCR3, CCR5 or CXCR4. At day 6 or at day 10, cultures were observed for syncytia formation and supernatants were analyzed for RT activity.

PCR and sequencing

DNA was isolated using the Qiagen DNA preparation kit (QIAGEN, Germany) according to the manufacturer's instructions. An approximately 300-bp region of *env* (including the V3 region) was amplified with a nested PCR approach using Pfu Turbo (Stratagene, USA) (Fig 1). The outer primers were JA167 (5'-TATCC/TTTTGAGCCAATTCCC/TATACA) and JA170 (5'- GTGATGTATTA/GCAA/GTAGAAAAATTC) and the inner primers were JA168 (5'-ACAATGC/TACACATGGAATTAA/GGCCA) and JA169 (5'-AGAAAAATTCC/TCCTCC/TACAATTAAA) (Leitner et al., 1996). The outer PCR was carried out as follows: one initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 15 s, 40°C for 15 s, and 72°C for 1 min and a final extension at 72°C for 5 min. The inner PCR protocol was as follows: an initial denaturation step of 94°C for 5 min, followed by 40 cycles of 94°C for 15 sec, 45°C for 15 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. Sequencing was done by using Big Dye Termination kit, (Applied biosystems; Foster City, USA) as described by the manufacturer (Fig 1), using 60 ng of DNA and primers JA168 and JA169. Sequences were analyzed using Chromas (http://www.technelysium.com.au/chromas.html), ClustalX (Thompson et al., 1997) and GeneDoc (http://www.psc.edu/biomed/genedoc). The entire approximately 300-bp region (including the V3 region) of *env* was analyzed. In Tables 1-4 we show the V3 region only.

Molecular cloning and sequencing of the V3 region

Proviral DNA derived from infected PBMC was amplified as described above. PCR products were A-tailed by incubation at 72°C for 10 min with 7.5 U of Taq polymerase (Invitrogen), 1.25 mM dATP and cloned using the Easy-T cloning kit (Promega Corporation, Madison, USA). Selected clones were amplified and sequenced as described above.

Sequence data

Sequences have been deposited in Genbank under accession numbers AY850200 to AY850217 (isolate 29); and AY850218 to AY850224 (isolate 31).

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Figure legends

Figure 1

Schematic picture of the selective biological cloning system A) First selection step: In the first selection step, virus isolates were diluted and passaged 1-3 times on U87.CD4-CCR3, -CCR5 and CXCR4 expressing cells. B) Second selection: Supernatants from first selection step were used to infect U87.CD4-CCR3, -CCR5 and –CXCR4-expressing cells in a second selection step. C) Genotypic determination: To confirm clonality V3 region from proviral DNA, obtained after second selection, was amplified and sequenced. D) Phenotypic determination: Confirmed biological clones were grown on PBMC and the phenotype was finally determined by infection of U87.CD4 cell lines.

Figure 2

Isolate 29 was diluted and passaged on U87.CD4-CCR3, -CCR5 and –CXCR4 expressing cells. Cultures were analyzed for syncytia formation and RT activity in supernatants on day 6 for CCR5- and CXCR4-expressing cells and on day 10 for CCR3-expressing cells. RT activity (a) and score of syncytia (b) in isolate 29 infected U87.CD4-CCR3, -CCR5, and -CXCR4 expressing cells. **(a)** Mean absorbance values for RT activity in supernatants from quadruplicate wells. **(b)** Mean score of syncytia in quadruplicate wells. Syncytia formation was scored as 3, large syncytia covering > 50% of the well; 2, large syncytia covering < 50% of the well; 1, ≤ 5 small occasional syncytia (LDSF).

Figure 3

Isolate 31 was diluted and passaged on U87.CD4-CCR3, -CCR5 and –CXCR4 expressing cells. Cultures were analyzed for syncytia formation and RT activity in supernatants on day 6 for CCR5- and CXCR4-expressing cells and on day 10 for CCR3-expressing cells. RT activity (a) and score of syncytia (b) in isolate 31 infected U87.CD4-CCR3, -CCR5, and -CXCR4 expressing cells. **(a)** Mean absorbance values for RT activity in supernatants from quadruplicate wells. **(b)** Mean score of syncytia in quadruplicate wells. Syncytia formation was scored as 3, large syncytia covering > 50% of the well; 2, large syncytia covering < 50% of the well; 1, ≤ 5 small occasional syncytia (LDSF).

Figure 4.

Nucleotide sequences corresponding to amino acids 13-25 of the V3 loop. A) Isolate 29

B) Biological clone 29A. C) Biological clone 29B. D) Heterogeneous sequence $29X_2$.

The arrow (↓) indicates heterogenic site.

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Table 1 Generation of biological clones, isolate 29

	First passage					
Clone[a]	**Cell Line**[b]	**Dilution of isolate**	**Second Selection**[c]	**Sequence**[d]	**Genotype**	**Phenotype**[e]
Isolate 29		Undiluted		CTRPNNNTRKGI??GPG?AF?AT??IIGDIRQAHC		R3R5X4
$29X_1$	CCR3	3^3	CCR3	Mixed		
$29X_2$			CCR5	Mixed		
$29X_3$			CXCR4	Mixed		
$29A_1$	CCR3	3^5	CCR3	----------SI---R--I--RK----------	I	R3R5X4
$29A_2$			CCR5	----------SI---R--I--RK----------	I	R3R5X4
$29A_3$			CXCR4	----------SI---R--I--RK----------	I	R3R5X4
$29B_1$	CCR3	3^6	CCR3	----------HM---R--YT-GN----------	II	R3R5
$29B_2$			CCR5	----------HM---R--YT-GN----------	II	R3R5
$29X_4$	CCR5	3^3	CCR3	Mixed		
$29X_5$			CCR5	Mixed		
$29X_6$			CXCR4	Mixed		
$29C_1$	CCR5	$4X3^5$	CCR3	----------SI---R--I--RK----------	I	R3R5X4
$29C_2$			CCR5	----------HM---R--YT-GN----------	II	R3R5
$29C_3$			CXCR4	----------SI---R--I--RK----------	I	R3R5X4
$29E_1$	CXCR4	3^1	CCR3	----------SI---R--I--RK----------	I	R3R5X4
$29E_2$			CCR5	----------SI---R--I--RK----------	I	R3R5X4
$29E_3$			CXCR4	----------SI---R--I--RK----------	I	R3R5X4
$29F_1$	CXCR4	3^6	CCR3	----------SI---R--I--RK----------	I	R3R5X4
$29F_2$			CCR5	----------SI---R--I--RK----------	I	R3R5X4
$29F_3$			CXCR4	----------SI---R--I--RK----------	I	R3R5X4

Table 1 continued

Isolate 29 was diluted and passaged on U87.CD4 cells expressing CCR3, CCR5 or CXCR4, followed by a second selection on the three U87.CD4 cell lines. DNA was isolated from cells in second selection, PCR amplified and the V3 region was sequenced.

a; Designation of clone, X represents a clone with heterogeneous V3 sequence.

b; Co-receptor expression of U87.CD4 cells used for first passage.

c; Co-receptor expression of U87.CD4 cells used in the second selection step.

d; Amino acid sequence of V3 loop ?, denotes heterogenic site. Mixed, denotes a non-clonal sequence.

e; Phenotype of biological clone.

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Table 2 Generation of biological clones, isolate 31

	First passage					
Clone[a]	**Cell Line**[b]	**Dilution of isolate**	**Second Selection**[c]	**Sequence**[d]	**Genotype**	**Phenotype**[e]
Isolate 31				CTRPNN?TRKRI?LGPGRVLYTTGEIIGDIRKAHC		R3R5X4
$31X_1$	CCR3	3^2	CCR3	Mixed		
$31X_2$			CCR5	Mixed		
$31X_3$			CXCR4	Mixed		
$31B_1$	CCR3	3^3	CCR3	------Y-----S----------------------	IV	R3R5X4
$31B_2$			CCR5	------Y-----S----------------------	IV	R3R5X4
$31B_3$			CXCR4	------Y-----S----------------------	IV	R3R5X4
$31X_4$	CCR5	$4X3^4$	CCR3	Mixed		
$31X_5$			CCR5	Mixed		
$31X_6$			CXCR4	Mixed		
$31E_1$	CCR5	$4X3^5$	CCR5	------N-----T----------------------	V	R5X4
$31E_2$			CXCR4	------N-----T----------------------	V	R5X4
$31X_7$	CXCR4	3^1	CCR3	Mixed		
$31X_8$			CCR5	Mixed		
$31X_9$			CXCR4	Mixed		
$31G_1$	CXCR4	3^6	CCR5	------N-----T----------------------	V	R5X4
$31G_2$			CXCR4	------N-----T----------------------	V	R5X4

Table 2 continued

Isolate 31 was diluted and passaged on U87.CD4 cells expressing CCR3, CCR5 or CXCR4, followed by a second selection on the three U87.CD4 cell lines. DNA was isolated from cells in second selection, PCR amplified and the V3 region was sequenced.

a; Designation of clone, X represents a clone with heterogeneous V3 sequence.

b; Co-receptor expression of U87.CD4 cells used for first passage.

c; Co-receptor expression of U87.CD4 cells used in the second selection step.

d; Amino acid sequence of V3 loop ?, denotes heterogenic site. Mixed, denotes a non-clonal sequence.

e; Phenotype of biological clone.

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Table 3 Generation of biological clones from isolate 29 by serial dilutions on CCR5-expressing cells

Clone[a]	Second Selection[b]	Sequence[c]	Genotype	Phenotype[d]
Isolate 29		CTRPNNNTRKGI??GPG?AF?AT??IIGDIRQAHC		R3R5X4
29a	CCR5	----------HM---K--Y--GN----------	III	R5
29b	CCR5	----------HM---K--Y--GN----------	III	R5
29c	CCR5	----------HM---K--Y--GN----------	III	R5
29d	CCR5	----------HM---K--Y--GN----------	III	(R3)R5

Isolate 29 was diluted and passaged three times on U87.CD4-CCR5 cells followed by a second selection on U87.CD4 cells expressing CCR3, CCR5 or CXCR4. The phenotype of biological clones isolated was determined and the V3 region was amplified and sequenced.

a; Designation of clone.

b; Cell line used in the second selection step.

c; V3 loop amino acid sequence of.

d; Phenotype of biological clone. Phenotype in parenthesis indicates weak coreceptor usage.

Table 4 Summary of phenotypic and genotypic differences

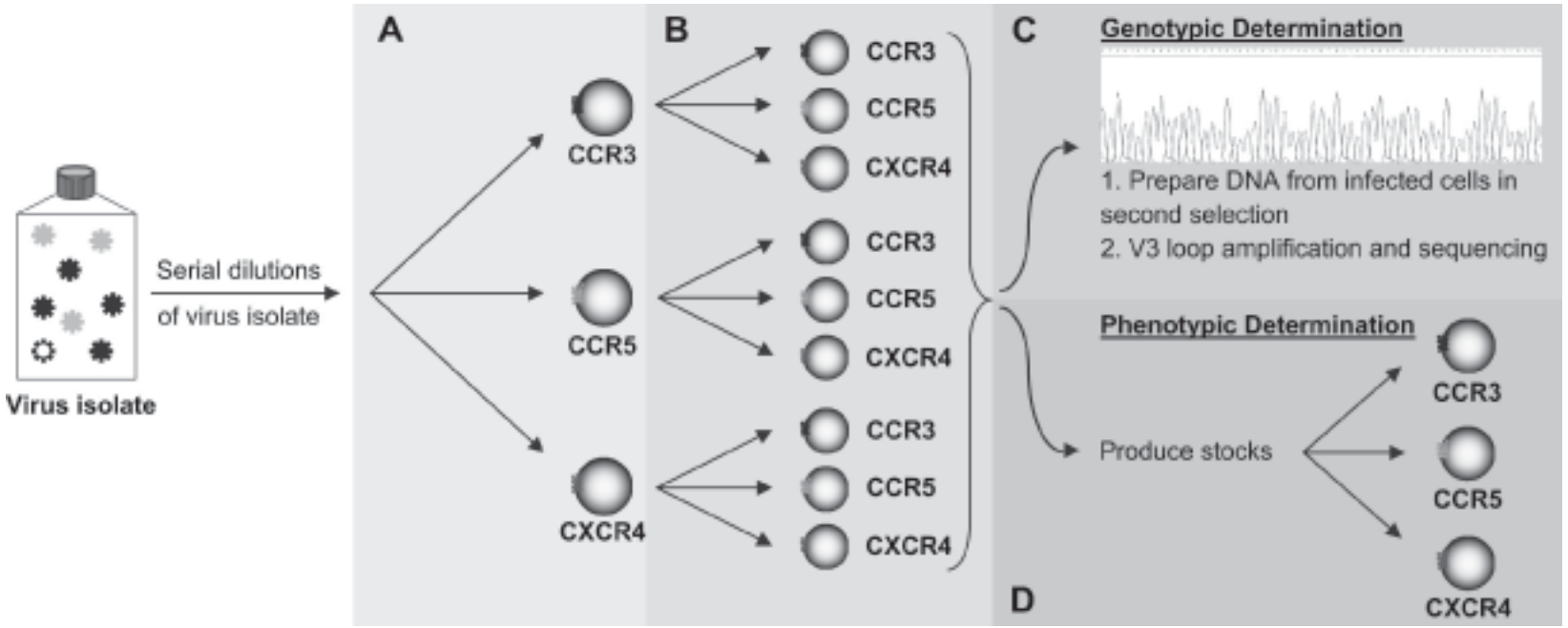
Number Of Clones	Phenotype[a]	Genotype[b]	Sequence[c]	Charge[d]
Isolate 29			CTRPNNNTRKGI??GPG?AF?AT??IIGDIRQAHC	
11	R3R5X4	I	----------SI---R--I--RK----------	+6
3	R3R5	II	----------HM---R--YT-GN----------	+4
3	R5	III	----------HM---K--Y--GN----------	+4
1	(R3)R5	III	----------HM---K--Y--GN----------	+4
Isolate 31			CTRPNN?TRKRI?LGPGRVLYTTGEIIGDIRKAHC	
3	R3R5X4	IV	------Y-----S----------	+5
6	R5X4	V	------N-----T----------	+5

a; Phenotype of biological clones. Phenotype in parenthesis indicates weak coreceptor usage.

b; Genotype of biological clones.

c; Amino acid sequence of the V3 loop.

d; Overall charge of the V3 loop.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Serial dilutions
of virus isolate
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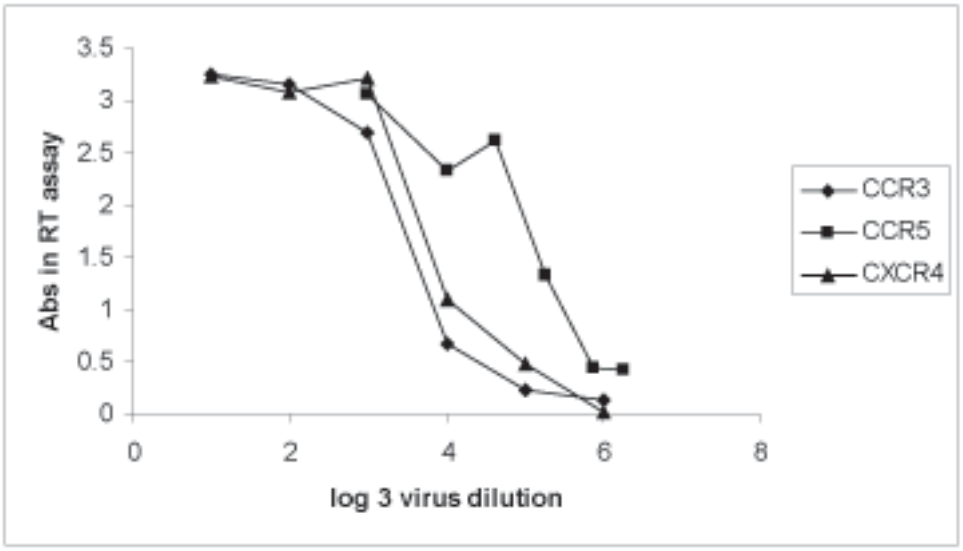
CCR5
CXCR4
B
CCR3
CCR5
CXCR4
CCR3
CCR5
CXCR4
CCR3
CCR5
CXCR4
C
Genotypic Determination
1. Prepare DNA from infected cells in second selection
2. V3 loop amplification and sequencing
Phenotypic Determination
Produce stocks
CCR3
CCR5
CXCR4
D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Abs in RT assay
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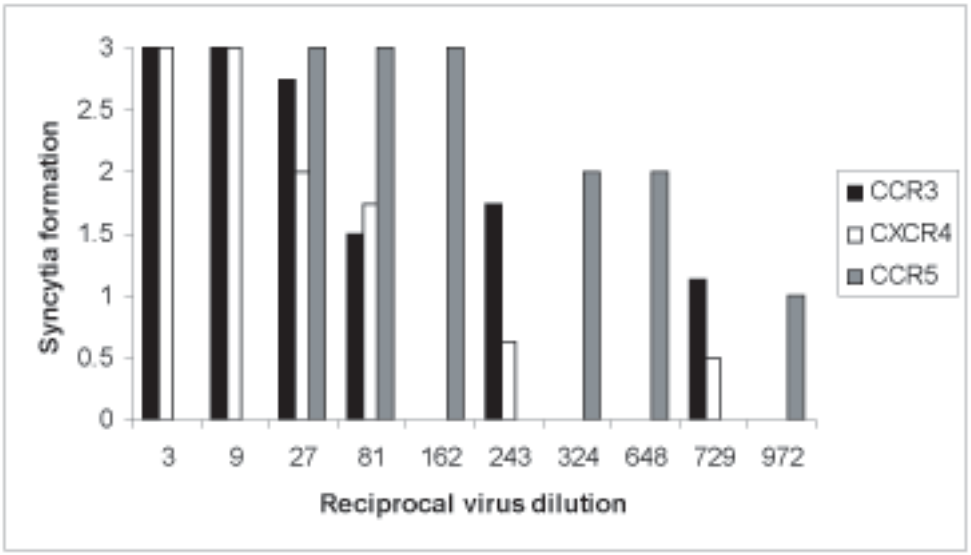
CCR3
CCR5
CXCR4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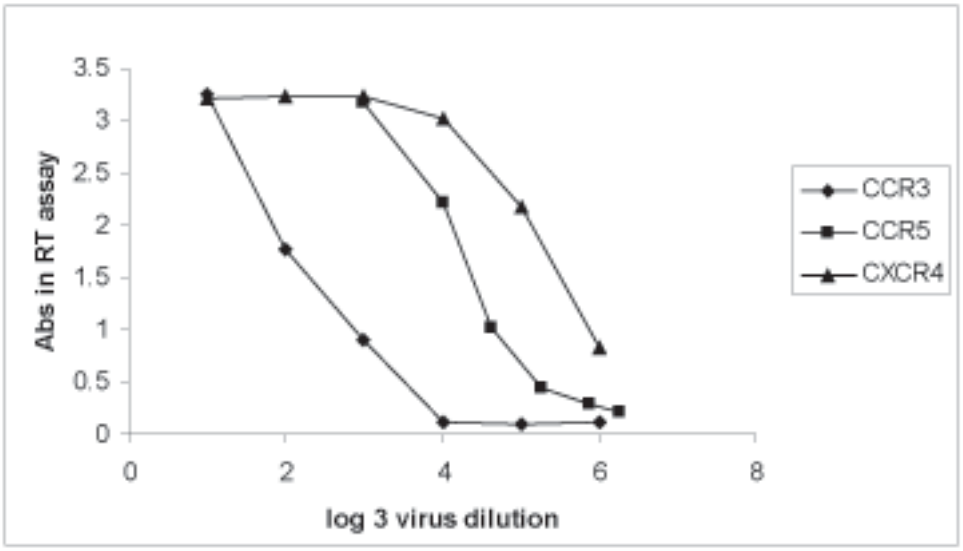
972
Reciprocal virus dilution
CCR3
CXCR4
CCR5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log 3 virus dilution
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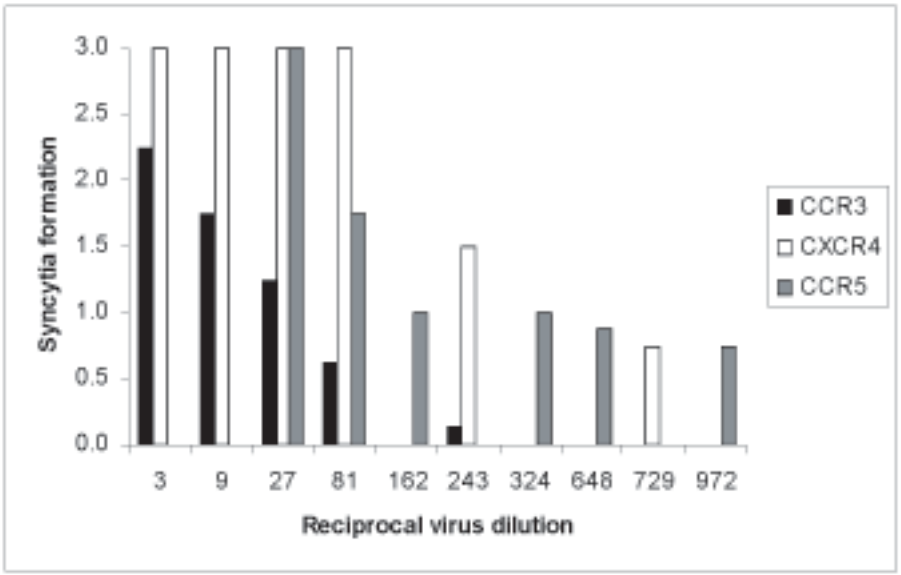
CCR5
CXCR4


Syncytia formation
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2.0
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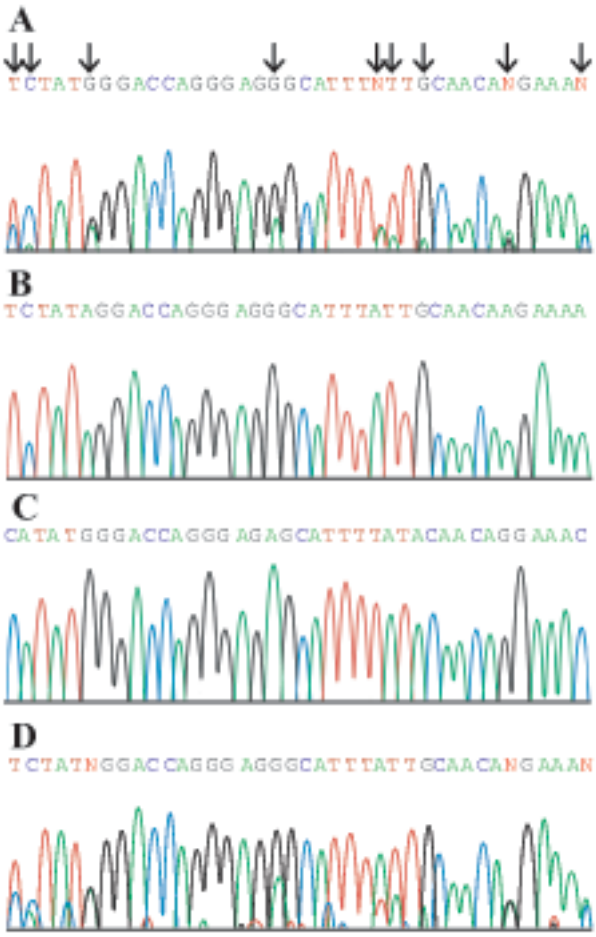
648
729
972
Reciprocal virus dilution
CCR3
CXCR4
CCR5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