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T20-insensitive HIV-1 from naïve patients exhibits high viral fitness in a novel dual-color competition assay on primary cells

Thomas Neumann[a], Isabel Hagmann[a], Sabine Lohrengel[a], Marintha L. Heil[b], Cynthia A. Derdeyn[b,c,1], Hans-Georg Kräusslich[a], Matthias T. Dittmar[a,*]

[a]*Department of Virology, Hygiene-Institute, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany*
[b]*Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA*
[c]*UAB Center for AIDS Research, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

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Abstract

The relationship between sensitivity to antiviral drugs and viral fitness is of paramount importance in understanding the long-term implications of clinical resistance. Here we report the development of a novel recombinant virus assay to study entry inhibitor-resistant HIV variants using a biologically relevant cell type, primary CD4 T-cells. We have modified the replication-competent molecular clone HIV_{NL4-3} to express a reporter protein (Renilla luciferase), Green Fluorescent Protein (EGFP), or Red Fluorescent Protein (DsRed2) upon infection, thus allowing quantification of replication. Luciferase-expressing virus was used to evaluate drug sensitivity, while co-infection with viruses carrying the green and red fluorescent proteins was employed in the competitive fitness assay.

Using envelope proteins from three T20 insensitive variants, lower levels of resistance were observed in primary CD4 T-cells than had been previously reported for cell lines. Importantly, dual-color competition assays demonstrated comparable or higher fitness for these variants despite their reduced T20 sensitivity. We conclude that reduced sensitivity to T20 is compatible with high viral fitness in the absence of selection pressure. Thus, simultaneously measuring both resistance and viral fitness using this newly described dual-color competition assay will likely provide important information about resistant viral variants that emerge during therapy with entry inhibitors.

Keywords: T20; HIV-1; Dual-color competition assay

Introduction

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) consists of two subunits, the outer surface unit (SU, gp120) and the transmembrane protein (TM, gp41). Together, these glycoproteins mediate virus entry into target cells. A detailed understanding of the structural changes that take place in the envelope protein during binding to CD4, coreceptor, and subsequently during membrane fusion has created new opportunities to prevent and treat HIV-1 infection. Recently, several compounds that target different steps of viral entry have entered clinical trials (for a review, see Clavel and Hance, 2004; Moore and Doms, 2003; Pierson and Doms, 2003). So far, only the fusion inhibitor T20 (also known as Enfuvirtide) which targets gp41 has been approved for clinical use and is currently included in combination antiretroviral treatment strategies (Greenberg and Cammack, 2004).

The transmembrane protein gp41 is the fusogenic component of the envelope/receptor complex. After tethering the virus particle to the target cell through the fusion peptide, conformational changes involving a membrane distal hydrophobic region (HR2) and a more proximal hydrophobic region (HR1) result in the formation of a six-helix bundle and concomitant shortening of the molecule. The change in free energy associated with this structural

* Corresponding author. Fax: +49 6221 565003.
E-mail address: Matthias.Dittmar@med.uni-heidelberg.de (M.T. Dittmar).
[1] Current address: Emory Vaccine Center, 954 Gatewood Road, Atlanta, GA 30329, USA.

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transition is considered to be sufficient to cause lipid mixing and membrane fusion. Since the T20 peptide is based on the sequence of the HR2 region from HIV-1_{LAI}, it is predicted to bind to a highly conserved hydrophobic groove located on the trimeric coiled coils of HR1, thereby preventing the formation of the six-helix bundle and inhibiting membrane fusion (Moore and Doms, 2003; Pierson and Doms, 2003).

The clinical benefits of antiretroviral treatment are limited by the selection of drug-resistant HIV-1 strains during therapy (Clavel and Hance, 2004; Nunberg et al., 1991; Richman, 1996). Databases of primary resistance mutations that emerged in the presence of reverse transcriptase inhibitor (RTI) and protease inhibitor (PI) therapy have been useful in predicting clinical resistance based solely on genotype. However, mutations in the reverse transcriptase (RT) and protease (PR) genes that confer drug resistance often also impair replication capacity (RC) (Blower et al., 2001; Brenner et al., 2002). This notion is supported by the observation that drug-susceptible variants rapidly outgrow the drug-resistant viral quasispecies in vivo after discontinuation of treatment. In addition, several in vitro studies have demonstrated a reduced overall performance of these enzymes when substituted with primary resistance mutations (Hance et al., 2001; Verhofstede et al., 1999). Secondary mutations can enhance drug resistance, restore replication capacity, or both. To determine viral fitness of drug-resistant variants, phenotypic assays have been developed that measure replication capacity in a single round infection assay as well as in a spreading infection (Parkin et al., 2002, 2004; Petropoulos et al., 2000). In the case of replication-competent viruses, parallel infections with different virus variants are difficult to quantitate, especially when only small differences are present and primary cells are used as target (Domingo et al., 1997). In vitro competition between two viruses (dual-infection/growth competition assay) using relevant target cells (such as PBMC) is expected to more accurately record relative fitness.

In vitro selection of T20-resistant HIV-1 variants by Rimsky et al. (1998) demonstrated the importance of the highly conserved, contiguous three amino acid residue sequence GIV at position 36–38 within the first heptad repeat region of gp41 (HR1) in the development of T20 resistance. Characterization of resistant viruses from phase II trials revealed similar changes to those observed in tissue culture, but indicated that the putative interaction site of T20 likely encompasses a larger region than previously considered, including residues 36–45 of HR1 (Poveda et al., 2002; Xu et al., 2002). Moreover, regions in the gp120 surface protein can also modulate sensitivity to T20 (Derdeyn et al., 2000, 2001; Reeves et al., 2002). In addition to coreceptor affinity, other steps within the entry process (attachment, CD4-binding, and the kinetics of the conformational changes within gp41) could also modulate the baseline susceptibility to T20. Investigations using viruses derived from T20-naive patients revealed a much wider range of T20 baseline sensitivity compared to similar studies using reverse transcriptase (RT) or protease (PR) inhibitors (Derdeyn et al., 2001; Hance et al., 2001; Labrosse et al., 2003; Verhofstede et al., 1999). This wide range of T20 sensitivity makes it difficult to define an absolute T20 therapy-induced resistance threshold. Since different regions of the envelope protein are likely to contribute to susceptibility to entry inhibitors, it is also not feasible to predict resistance based on viral genotyping alone. Therefore, the development of phenotypic assays to assess entry inhibitor resistance and viral fitness is of great importance. Our group and others have demonstrated that recombinant virus assays (RVAs) can be used for measuring phenotypic resistance (Dittmar et al., 2001; Fikkert et al., 2002; Trouplin et al., 2001). However, due to the complex interactions between distal regions of Env, RVAs that incorporate the entire *env* gene of drug-naive or T20-treated patients will be required to produce meaningful data regarding resistance and fitness. Furthermore, infection of T-cells most closely reflects the in vivo situation and is therefore necessary to validate results obtained in cell lines or indicator cells. To directly assess viral fitness of T20-resistant virus relative to a sensitive strain, growth competition assays will be important (Lu and Kuritzkes, 2001; Quinones-Mateu et al., 2000, 2002; Rangel et al., 2003). These assays are beneficial in the context of evaluating baseline virus from drug-naive patients, or after T20-treatment failure to quantify the clinical benefit of this treatment regimen. With regards to T20 treatment, a comprehensive data set is slowly emerging. Recently, Lu et al. reported that the viral fitness of HIV_{NL4-3} variants encoding T20 selected point mutations in HR1 as well as full-length post-treatment patient-derived envelope genes showed reduced fitness (Lu et al., 2004). Nevertheless, more information between the interplay of drug resistance and viral fitness is needed to assess the long-term usefulness of fusion inhibitor therapy.

Here we analyzed envelope proteins from virus variants with reduced T20 sensitivity obtained from three T20-naive patients. We observed lower levels of resistance than had been previously reported for the same variants analyzed in cell lines. Importantly, dual-color competition assays (DCCAs) with primary CD4 T-cells as targets demonstrated comparable or higher fitness of these variants compared to a coreceptor-matched subtype B reference virus. We conclude that reduced sensitivity to T20 is compatible with high viral fitness in the absence of drug selection pressure. By measuring both drug sensitivity and viral fitness, the relative cost of resistance to entry inhibitors can be better quantified.

Results

Replication kinetics of the novel marker viruses are similar to those observed for wild-type HIV-1_{NL4-3}

One goal of this study was to establish a combined resistance/viral fitness assay using primary T-cells as targets to characterize Envs before therapy and during treatment

with entry and fusion inhibitors. Therefore, three different recombinant marker viruses were constructed. The vectors were designed for directional insertion of the env gene of interest through use of two unique restriction sites present in the env-deleted proviral constructs (Fig. 1A). We partially removed the nef open reading frame and cloned the genes for renilla luciferase, EGFP, and DsRed2 into a derivative of the proviral clone pNL4-3 resulting in TN7Δ, TN6GΔ, and

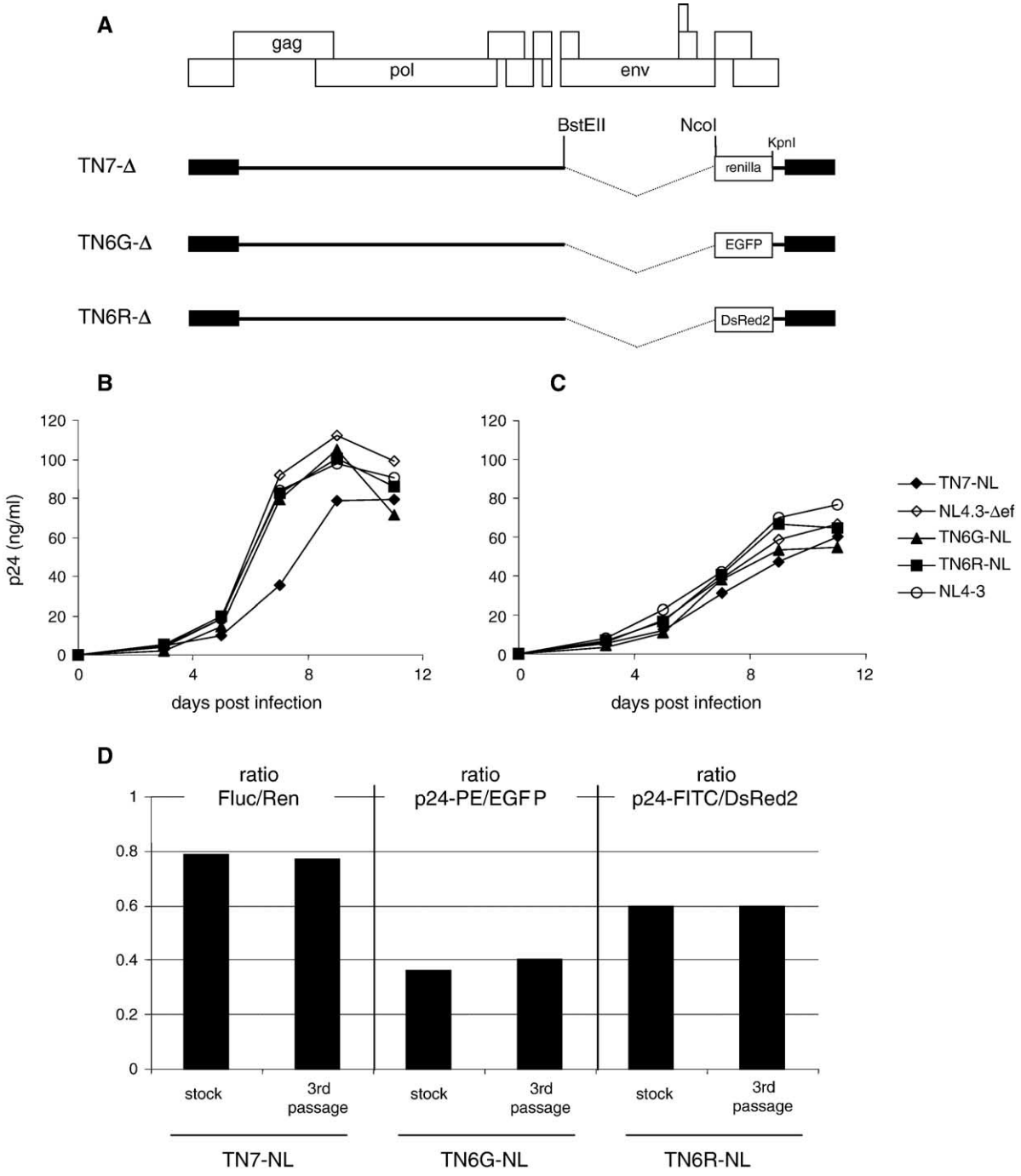

Fig. 1. (A) Schematic representation of the envelope-deleted marker virus plasmids TN7, TN6G, and TN6R. These three plasmids encode for renilla luciferase (TN7), EGFP (TN6G), and DsRed2 (TN6R) in the place of the nef gene. Insertion of envelope genes from different patient samples after PCR amplification was accomplished by restriction with *Bst*EII and *Nco*I, resulting in full-length replication competent proviral plasmids. (B and C) Growth of wild-type NL4.3 and NL4.3Δnef in comparison to the marker viruses expressing the NL4-3 Env (TN7-NL, TN6G-NL and TN6R-NL) in PM1 cells (B) and PHA/IL-2 stimulated PBMC (C). Target cells were infected with an MOI = 0.001 and monitored over time for p24 antigen release into the supernatant. (D) The marker genes (renilla luciferase, EGFP, DsRed2) are stable after serial passage on PM1 cells. After three consecutive passages in PM1 cells, the ratio of intracellular p24 (detected using a fluorophore-conjugated anti-p24 antibody) to EGFP or DsRed2 expression was compared to the original stock virus generated by 293T transfection. To evaluate the stability of renilla luciferase expression (TN7-NL), the indicator cell line TZM-bl was infected with passaged and original stock virus and the ratio of (tat-induced) firefly to renilla luciferase activity was determined and compared.

TN6RΔ, respectively. All three marker viruses combined allow for the characterization of the envelope protein of interest in terms of resistance and viral fitness compared to standard or baseline HIV-1 envelopes in a primary T-cell culture system.

We first investigated whether expression of the marker genes (EGFP, DsRed2, and renilla luciferase) affected the replication capacity of recombinant marker viruses expressing the env gene of NL4-3 in the T-cell line PM1 or in primary T-cells. Infection of PM1 cells with the marker viruses TN6G-NL and TN6R-NL resulted in replication curves similar to that of wild-type and nef-deleted NL4-3 viruses (Fig. 1B). TN7-NL displayed a slight delay in replication kinetics on PM1 cells, but this phenotype was not observed in primary T-cells (Fig. 1C), where all three marker viruses replicated with kinetics similar to the control viruses.

To perform viral fitness assays, the marker viruses must stably express the reporter genes over several rounds of replication. Therefore, each reporter virus was serially passaged three times on PM1 cells and reporter gene expression of the passage 3 virus was compared to the original stock (Fig. 1D).

In the case of TN6G-NL and TN6R-NL, the relative ratio of viral protein expression and marker fluorescence intensity was analyzed. Cells were fixed and stained for intracellular expression of the HIV-1 capsid protein p24 using anti-p24 antibodies conjugated with phycoerythrin (for TN6G-NL) or FITC (for TN6R-NL). Subsequently, cells were analyzed by FACS and the ratio of cells expressing p24 and marker protein was determined. Fig. 1D shows that this ratio was very similar when infections with the transfection-derived stock virus or with passage 3 virus were compared, indicating that the marker gene was stably maintained. A similar result was observed for TN7-NL. In this case, the relative activity of firefly luciferase (induced by infection of TZM-bl cells) and renilla luciferase activity (encoded by the reporter virus) was quantified, and the ratio was shown to be constant over 3 passages. All three reporter viruses therefore maintain stable expression of EGFP, DsRed2, and renilla luciferase after multiple passages in PM1 cells.

T20-insensitive envelope proteins expressed by the marker virus TN7 display similar inhibition profiles on TZM-bl cells and PBMC

It has been shown that entry inhibitor resistance can be defined by infecting indicator cell lines, such as TZM-bl (Wei et al., 2002) and CEM-NKR (Spenlehauer et al., 2001), with viral isolates or Env pseudotypes. We therefore determined the phenotypes of three previously characterized T20-insensitive envelopes from three different patients (Heil et al., 2004) using our luciferase reporter backbone TN7Δ and the indicator cell line TZM-bl.

These env genes (R14, X10, and X23) were derived from a large panel of isolates from T20-naive patients and are representative of the wide range of T20 responsiveness that has been described (Derdeyn et al., 2000, 2001; Labrosse et al., 2003). The HR1 sequence of these Envs does not contain amino acids in positions 36–45 that are known to confer T20 resistance, although the L45M variation in X10 has been associated with T20 resistance when present in combination with other mutations (Table 1). After inserting these three envelope genes into TN7Δ, 293T cells were transfected and recombinant virus harvested and titrated on TZM-bl cells.

Recombinant viruses encoding for renilla luciferase (TN7) and expressing Envs from the X4-tropic virus NL4-3 (NL) and the R5-tropic virus Yu2-(Yu2) were used for comparison. Fig. 2A shows the inhibition of infection by the five recombinant viruses in TZM-bl cells. Infection in the absence of T20 was set to 100% and relative infection in the presence of increasing concentrations of T20 is depicted. This experiment demonstrated that viruses carrying the R14, X10, and X23 Env required higher concentrations of T20 for inhibition than observed for the reference viruses (Fig. 2A). IC_{50} values were determined for all five viruses (Table 2) and the relative insensitivity was calculated. These results confirmed the relative order of T20 insensitivity of these

Table 1
Amino acid sequence of heptad repeats 1 and 2 (HR-1 and HR-2) of the standard viruses NL4.3 and Yu2 and the T20-insensitive viruses R14, X10, and X23

```
HR1
CON_B     QaRqllsGIvqQqnnLlrAieaQqhllqlTvwgiKQLqarvLaverYLkdqqLlg
NL        QARQLLSDIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARVLAVERYLKDQQLLG
Yu2       -------G----------------------------------------R------
R14       ---L---G----------------M-----------------------------
X10       ---L---G--------M-----------------------L-------R------
X23       ---L---G-----------------M-----------------L----Q------

HR2
CON_B     wdnmTwmewereidnytsliytLieesqnqQekNeqeLleldkwaslwnwfd
NL        WENMTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFN
Yu2       -D-----K-E---D---HI-Y----Q-------------A-----------D
R14       -DK------EK—-D---G—-YD-LA----------K------------D---
X10       --------------------YN-L------------D--A-----N-----D
X23       -D----K—-E---D-V— I-YT-LG-----------------R-----S--D
```

A consensus_B sequence is included as reference (Con_B; residues in upper cases are invariant in subtype B viruses). Note that the T20-insensitive viruses R14, X10, and X23 do not encode for residues in HR-1 known to confer T20 resistance (location of resistance mutations are underlined for NL sequence).

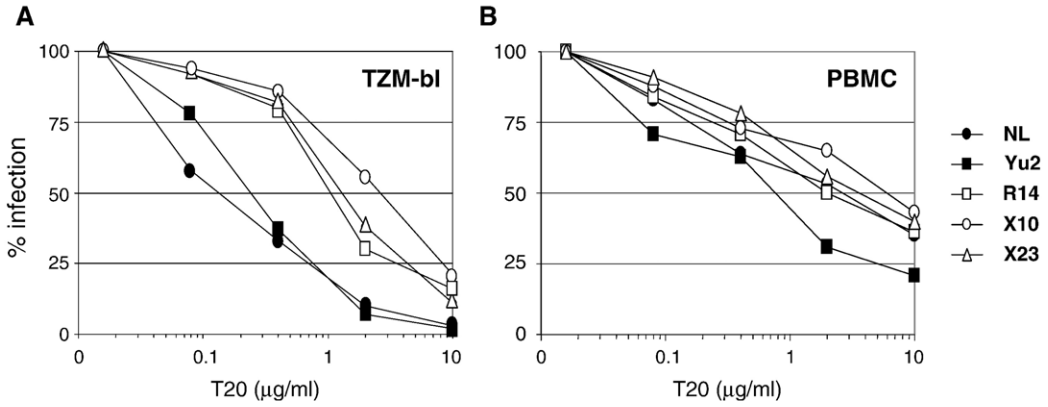

Fig. 2. Sensitivity of patient-derived envelope proteins to the fusion inhibitor T20. The envelope genes R14, X10, and X23 were derived from T20-naive HIV-positive individuals and have been described previously (Derdeyn et al., 2001; Heil et al., 2004). These envelope genes were cloned into TN7 and these viruses were used to infect TZM-bl indicator cells (A) or PBMC (B). Reference viruses were constructed that express either the envelope of NL4-3 (TN7-NL) or Yu2 (TN7-Yu2). (A) TZM-bl cells were infected with 1000 IU/well in the presence of increasing concentrations of T20 (0.02–10 µg/ml). Renilla luciferase activity was measured 48 h post-infection and is plotted on the *y*-axis as the percent infection relative to control infection (without fusion inhibitor). These curves were used to calculate IC_{50} values (see Table 2) for T20 for each envelope protein. Shown are the means of triplicate infections for each inhibitor concentration. (B) CD8-depleted PBMCs were infected with 5000 IU/well using the concentrations of T20 described above. The percent infection relative to control infections without fusion inhibitor is plotted against fusion inhibitor concentration. These data were used to calculate IC_{50} values for T20 for each envelope (see Table 2). Shown are the means of triplicate infections for each inhibitor concentration.

three Envs (Heil et al., 2004). The three T20-insensitive Envs confer a 7- to 14-fold lower sensitivity to T20 when compared with the control viruses TN7-NL and TN7-Yu2. These results were obtained through the detection of renilla luciferase activity due to viral infection by the marker virus TN7. The induction of firefly luciferase in TZM-bl cells upon infection by these viruses resulted in similar inhibition curves and changes in relative T20 insensitivity (data not shown).

We next characterized the sensitivity to T20 upon infection of primary T-cells (Fig. 2B). As described for the TZM-bl experiments, primary T-cells were infected in the presence of increasing concentrations of T20 and the relative rate of infection was calculated from the renilla luciferase activities obtained after 3 days. IC_{50} values for all viruses tested were generally higher, including the control viruses (IC_{50} between 800ng/ml to 6500ng/ml) in comparison to the data obtained from TZM-bl infections. Importantly, the relative order of resistance was preserved, but the relative differences were much less pronounced. This is best seen when looking at the fold change (Table 2), which ranges from 7.5- to 14-fold in TZM-bl cells and from 2.5- to 8-fold in primary T-cells.

Table 2
Evaluation of T20 sensitivity in TZM-bl indicator cells and PBMC relative to reference strains

Env	Coreceptor	T20 IC_{50} (ng/ml)		Relative T20 insensitivity	
		TZM-bl	PBMC	TZM-bl	PBMC
NL	X4	180 ± 30	1500 ± 80	1	1
Yu2	R5	200 ± 20	800 ± 55	1	1
R14	R5	1550 ± 100	2000 ± 50	7.5	2.5
X10	R5	2800 ± 90	6500 ± 50	14	8.1
X23	R5X4	1720 ± 110	3200 ± 120	9	2.0–4.0

The mean IC_{50} values of the TN7 vector expressing envelope proteins NL, Yu2, R14, X10, and X23 were calculated from at least four independent TZM-bl and PBMC infections. Coreceptor usage for the T20-insensitive Envs was determined using GHOST indicator cells (data not shown) and is indicated in the second column. Relative T20 sensitivity was calculated by dividing the IC_{50} value for each T20-insensitive Env by the IC_{50} value for TN7-NL or TN7-Yu2, respectively (depending on coreceptor usage). Accordingly, the dual-tropic envelope X23 has been attributed two values.

Dual-color competition assay in PM1 cells and PBMC

Reduced viral fitness contributes to the continued benefit of antiviral therapy despite the presence of drug resistance. In the case of reverse transcriptase and protease inhibitors, drug resistance is associated with a reduction in viral fitness (Quinones-Mateu et al., 2002; Rangel et al., 2003).

To establish ‘proof of concept’ for the dual-color competition assay, PM1 cells were infected (MOI = 0.025) with marker viruses encoding for the wild-type NL4-3 envelope protein and an envelope protein encoding a single amino acid change at position 38 of gp41 (V38A: GIA), known to confer resistance to T20 in vitro and in vivo. Cells were harvested at day 3 and day 7 post-infection with TN6G-NL and TN6R-GIA and total EGFP and DsRed2 fluorescence (at 508 nm and 582 nm, respectively) was determined using a fluorometer. Fig. 3 illustrates one example of the viral fitness data obtained. Since the ratio of green fluorescence to red fluorescence changed between day 3 and day 7 for both marker viruses (e.g., TN6G-NL/TN6R-NL and TN6G-GIA/TN6R-GIA), a neutral zone was defined for each experiment and each time point (grey area in Fig. 3, see also Figs. 4B and C). A difference in viral fitness was only considered valid if both time points revealed a growth advantage for one envelope variant over the other. The dual infection of PM1 cells with the two

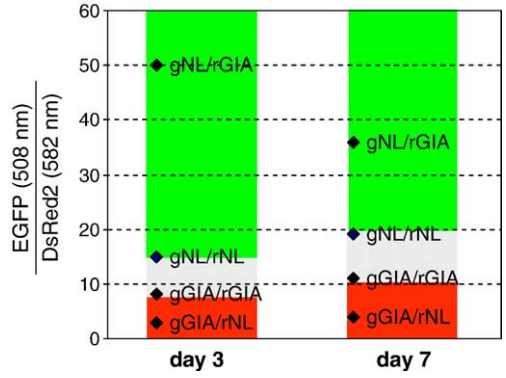

Fig. 3. Relative fitness of TN6G and TN6R marker viruses expressing the reference envelope NL compared to GIA, an envelope protein encoding for a single point mutation at position 38 (V38A: GIA), known to confer resistance to T20 in vitro and in vivo. A dual infection/growth competition assay was performed on PM1 cells. PM1 cells were infected at an MOI of 0.025 with competing viruses expressing EGFP or DsRed2. Representative results are shown. The 'neutral' zone is shown in grey. Only EGFP/DsRed2 fluorescence ratios calculated above or below this 'neutral' zone are considered as the EGFP virus having a growth advantage over the DsRed2 virus or vice versa. In addition, the EGFP/DsRed2 fluorescence ratio must remain above the neutral zone at both time points (day 3 and day 7) for the EGFP expressing virus to be considered as having a true growth advantage over the DsRed2 expressing virus. These results were confirmed by switching the marker genes between a certain pair of viruses and the ratio (DsRed2 vs. EGFP) for both days falling into the 'red' zone.

marker viruses TN6G-NL and TN6R-GIA resulted in a green to red fluorescence ratio of 50 at day 3 and 35 at day 7, significantly outside the 'neutral zone' (ratio between 8 and 20), indicating a growth advantage of TN6G-NL. The opposite pair of viruses was analyzed as well (TN6G-GIA and TN6R-NL), and resulted in green to red fluorescence ratios of 3 and 4 for day 3 and day 7 post-infection. These ratios were again outside the defined 'neutral zone'. Therefore, both combinations of envelope genes were tested and for both dual-color competition assays the recombinant virus encoding the wild-type envelope protein of NL4-3 scored as 'more fit' compared to the recombinant virus encoding the T20-resistant envelope protein GIA, confirming results by Lu et al. (2004).

Next, a potential correlation between T20 baseline insensitivity and viral fitness was analyzed. To this end, the dual-color competition assays were performed in primary T-cells using the EGFP and DsRed2 marker viruses expressing T20-insensitive Envs or reference Envs. This provided a direct comparison of viral fitness relative to the more sensitive NL4-3 and Yu2 recombinant viruses in a more relevant primary cell system.

Initially, viral input and the time course of fitness measurement were optimized for the dual-color competition assay in PBMC. It had been shown that high viral input results in dually infected cells (Dang et al., 2004) and such cells are the source for recombination. Therefore, PBMCs were infected at a low infectious dose of virus (MOI = 0.001, titrated on PBMC) to minimize the potential for recombination between the two viruses investigated. This infectious dose resulted in 5–15% infected cells on day 4 and 10–25% infected cells on day 8 (depending on the donor PBMC) with no double-positive cells detected by FACS analysis. These two time points were chosen for fitness measurements because sufficient cell numbers were detectable on day 4 and increasing numbers of dead cells were apparent at time points later than day 8.

Fig. 4A illustrates an example of the viral fitness data obtained. The dual infection of PBMC with TN6G-X10 and TN6R-X10 (gX10/rX10) resulted in a green fluorescence to red fluorescence ratio of 3.5 at day 4 and 2.8 at day 8. The dual infection using TN6G-X10 and TN6R-NL (gX10/rNL), however, resulted in a fluorescence ratio of 3.5 at day 4 and increased to 8.5 at day 8, indicating a growth advantage of the recombinant virus encoding the X10 Env. This result was confirmed by the dual infection using the reverse pair of marker-protein-expressing viruses. The dual infection using TN6G-NL and TN6R-X10 (gNL/rX10) resulted in a fluorescent ratio of 0.2 for day 4 and day 8, indicating a growth advantage of the marker virus TN6R-X10. Again, the ratio of green fluorescent to red fluorescent cells changed between day 4 and day 8 for marker viruses expressing the same *env* gene (e.g., TN6G-X10/TN6R-X10 and TN6G-NL/TN6R-NL), therefore a neutral zone was defined for each FACS-based experiment and each time point (grey area in Figs. 4B and C) as well. A difference in viral fitness was only considered valid if both time points revealed a growth advantage for one envelope variant over the other (boxed pairs in Figs. 4B and C). In addition, due to differences in the maturation time between EGFP and DsRed2, and the residual green fluorescence of the mature DsRed2 protein, both combinations of envelope genes were tested and scored positive only if both combinations yielded the same results (Fig. 4A for envelope protein X10).

All possible combinations were tested in this assay and significant differences were observed for viruses carrying the X10 env gene against the respective reference viruses. For both time points, the 'green' marker virus expressing the X10 envelope was more fit compared to TN6R-NL, the DsRed2 expressing control virus. Infections using the reverse pair of marker viruses and envelope proteins revealed again that TN6R-X10 was more fit than TN6G-NL (Fig. 4). Similar results were obtained when the dual infections were carried out using marker viruses expressing X10 envelope and Yu2 envelope, respectively (Fig. 4C). Surprisingly, the X10 envelope carrying marker virus efficiently out-competed viruses carrying either the NL4-3 or the Yu2 envelope proteins, although these are inhibited by concentrations of T20 that are 4-fold and 8-fold less than for X10 env carrying recombinant viruses. The other T20-insensitive envelopes, R14 and X23, were similar in fitness compared to the control viruses expressing NL4-3 and Yu2 envelopes (Figs. 4B and C) when expressed by the novel color marker viruses despite their 2-fold greater resistance against T20.

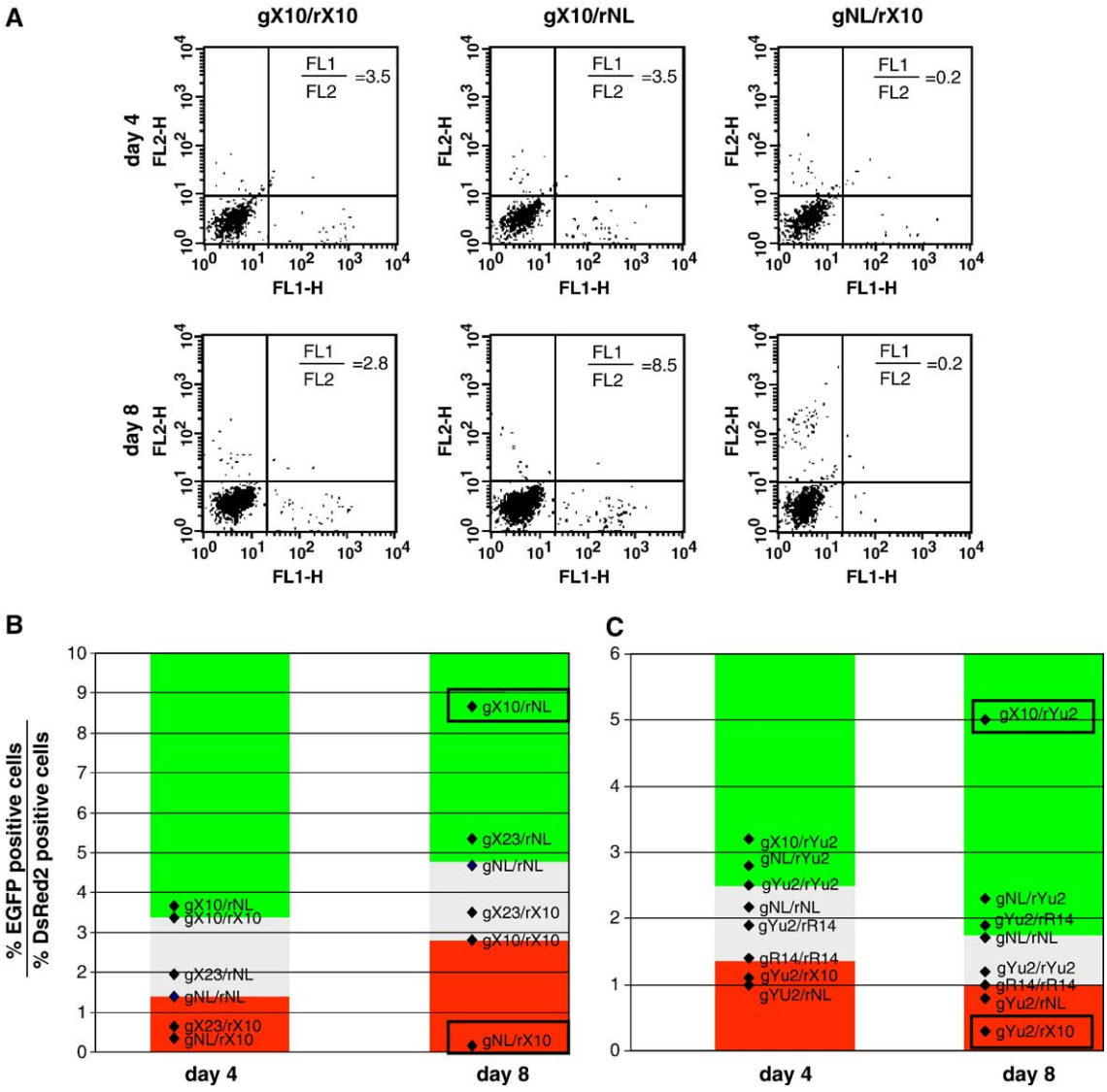

Fig. 4. Relative fitness of TN6G and TN6R marker viruses expressing the reference envelopes NL and Yu2 compared to patient-derived envelopes R14, X10, and X23. A dual infection/growth competition assay was performed on CD8-depleted PBMC. PBMCs were infected at an MOI of 0.001 with competing viruses expressing EGFP or DsRed2. (A) Percentage of fluorescent cells was measured at day 4 and day 8 post-infection with TN6G-X10/TN6R-X10, TN6G-X10/TN6R-NL, and TN6G-NL/TN6R-X10, respectively. The ratio of EGFP to DsRed2 (FL1/FL2) was determined at both time points. (B and C) A dual infection/growth competition assay was performed on CD8-depleted PBMC. PBMCs were infected at an MOI of 0.001 of competing virus pairs expressing EGFP or DsRed2. Representative results from two independent infections of PBMC (done in triplicate) are shown. These infections resulted in 5–15% infected cells at day 4 and 10–25% infected cells at day 8. The 'neutral' zone is shown in grey. Only EGFP/DsRed2 ratios calculated above this 'neutral' zone are considered as the EGFP virus having a growth advantage over the DsRed2 virus. In addition, the EGFP/DsRed2 ratio must remain above the neutral zone at both time points (day 4 and day 8) for the EGFP-expressing virus to be considered as having a true growth advantage over the DsRed2-expressing virus. These results were confirmed by switching the marker genes between a certain pair of viruses and the ratio (DsRed2 vs. EGFP) for both days falling into the 'red' zone. The boxed pairs for B and C indicate the pairs with the highest relative fitness score observed.

To summarize the data in Fig. 4 and data not shown, we calculated the relative fitness of each marker virus encoding for the different envelope proteins in relation to the standard viruses encoding for the envelope proteins NL and Yu2, respectively (Table 3). This resulted in the establishment of an order of viral fitness for the envelope expressing marker viruses that were studied:

$$R14 = Yu2 < NL4.3 = X23 < X10$$

Discussion

Here we describe novel marker viruses that can be used for quantification of entry inhibitor resistance in conjunction with true viral fitness measurements using a dual-color competition assay (DCCA). Furthermore, these parameters can be evaluated using biologically relevant target cells such as primary CD4 T-cells, rather than indicator cells or T cell lines as described before (Dittmar et al., 2001; Fikkert et al., 2002; Lu and Kuritzkes, 2001; Spenlehauer et al., 2001;

Table 3
Relative fitness of recombinant viruses carrying T20-insensitive Envs

	Relative fitness (RF)	
	Relative to NL	Relative to Yu2
NL	1.0	1.4 ± 0.1
Yu2	0.7 ± 0.2	1.0
R14	n.d.	0.9 ± 0.1
X10	2.5 ± 0.3	3.0 ± 0.4
X23	1.8 ± 0.3	1.2 ± 0.1

The dual infection/growth competition assay was used to compare the relative fitness of each patient-derived Env to NL4-3 (X10 and X23) or Yu2 (R14, X10, X23). The relative viral fitness was calculated as fold difference between the ratio of green to red fluorescence (FL1/FL2 ratio) of a given envelope variant and the mean FL1/FL2 ratio of the neutral zone at day 8 post-infection (see Figs. 3B and C). The relative fitness was calculated from four independent experiments (n.d. = not done).

Trouplin et al., 2001; Wei et al., 2002). The exchange of the nef gene for either the renilla luciferase gene or one of the two fluorescent proteins, EGFP and DsRed2, respectively, resulted in replication-competent viruses that had replication kinetics comparable to both wild type and *nef*-deleted NL4-3 (Figs. 1B and C). This result was a critical prerequisite to establish the dual infection/growth competition assay. A second prerequisite was that the marker gene be stably expressed over multiple rounds of replication. All three marker genes were stably expressed after three passages in PM-1 cells (Fig. 1D). Finally, the detection of all three marker genes requires limited sample preparation (renilla luciferase: lysis of cells and transfer to a luminometer; EGFP and DsRed2: fixation of cells and transfer to FACScan or fluorometer) and all measurements can be performed with high throughput. Thus, this reporter virus system is well suited to study the links between entry inhibitor resistance and viral fitness, allowing quantitative luciferase activity measurements in primary T-cells as well as efficient and quantitative measurement of viral spread in dually infected cultures using FACS or fluorometer analysis.

Several envelope-deleted marker viruses that could be used to study viral sensitivity to entry inhibitors have been described (Connor et al., 1995; He et al., 1997). Our intention was to develop a system in which viral fitness could be directly compared with sensitivity to entry inhibitors. Here we inserted three patient-derived *env* genes from viruses with varying levels of T20 and T649 sensitivity into each of the three marker viruses to simultaneously assess T20 sensitivity and viral fitness relative to a reference virus. IC_{50} values similar to those previously described (Derdeyn et al., 2001; Heil et al., 2004) were obtained for each recombinant virus (Table 2). Higher IC_{50} values were obtained in PBMC compared to cell lines, which is consistent with some previous studies (Ketas et al., 2003), but not others (Derdeyn et al., 2000, 2001). However, drug sensitivity assays using both TZM-bl and PBMC as target cells resulted in the same order of sensitivity to T20, with X10 envelope carrying recombinant virus being the least sensitive. Importantly, the estimated fold change (FC) or relative T20 insensitivity for the recombinant viruses carrying T20-insensitive envelopes relative to the reference viruses was smaller on PBMC than on TZM-bl indicator cells. This result indicates a potential overestimation of emerging entry inhibitor resistance when cell lines are used. However, greater numbers of pre-treatment and resistant envelope proteins expressed by the recombinant marker viruses need to be studied to clarify this finding.

The dual-color competition assay on PM1 cells using EGFP and DsRed2 expressing recombinant viruses encoding for wild type NL4-3 envelope and the known T20 resistant envelope GIA (Fig. 3) confirmed previous data regarding the reduced viral fitness of the GIA variant (Lu et al., 2004). Furthermore, this result provided proof of concept for this fluorescence-based assay to yield meaningful data. The dual-color competition assay on primary T-cells described here in detail resulted in the determination of a relative viral fitness order for three recombinant viruses carrying T20-insensitive envelope proteins (R14, X10, X23) in comparison to two reference viruses (NL4-3, Yu2). Many assays to determine replication capacity have used cell lines rather than primary cells (Prado et al., 2002; Resch et al., 2002; Simon et al., 2003; Trkola et al., 2003; Zhang et al., 2004). In the case of protease inhibitors (PI), it has been shown that the metabolism and/or export of PIs from the cytoplasm differ between T cell lines and primary T-cells (Kim, 2003). These observations argue that more biologically relevant systems, such as the PBMC-based viral fitness assay described here, will be useful in studying entry inhibitor resistance and its consequences on viral fitness. However, one has to keep in mind that all recombinant virus assays analyze the phenotype of RT, protease, or the envelope protein after cloning the respective genes into modified marker virus plasmids. The interaction of patient-derived envelope protein and patient-derived gag, for example, cannot be addressed and might contribute to viral fitness as well.

Reference envelope proteins, such as the well-characterized envelope proteins of NL4-3 and Yu2, are useful to facilitate the validation and comparison of data generated from different studies. In the present study, the recombinant virus carrying the X10 envelope gene has the greatest level of relative fitness, although this envelope confers the most insensitive T20 phenotype, with an IC_{50} 8-fold higher than that required to inhibit NL4-3 on PBMC. The other two T20-insensitive envelope proteins R14 and X23 cloned into the marker viruses did not show a substantial growth advantage over the reference viruses NL4-3 and Yu2, but did not show a reduction in fitness either. Interestingly, the X23 Env confers a 70-fold less sensitive phenotype on TZM-bl cells to a related fusion inhibitor, T649, compared to NL4-3 (Heil et al., 2004), yet these two recombinant viruses exhibit a similar level of viral fitness. In the present study, an inverse correlation between fusion inhibitor insensitivity and viral fitness was not observed.

For the fusion inhibitor T20, our results indicate that the envelope protein can accommodate baseline insensitivity,

and potentially some forms of resistance, without a reduction in replication fitness. Such variants with considerable primary insensitivity against T20 (i) may contain a rather low genetic barrier for T20-induced resistance at least in some patients or (ii) may be able compensate T20 treatment-induced loss of viral fitness. Highly insensitive Envs from naive patients could therefore contribute to the selection of T20-resistant variants without a significant fitness cost. This has been shown for RTI and PI selected variants (Nijhuis et al., 2001), where secondary mutations compensated for a reduction in fitness. Therefore, additional experiments using T20-resistant envelope proteins obtained from long-term-treated patients (likely to have developed secondary mutations) are needed to clarify whether compensatory mutations are detectable and can restore fitness once T20 resistance has developed.

However, clinical trials revealed that growth of T20-resistant virus was impaired (Greenberg and Cammack, 2004). Recently, Lu et al. determined the fitness of HIV_{NL4-3} variants encoding for T20 selected amino acid residues in HR1 found in treated patients. Using a growth competition assay, they confirmed a reduced viral fitness of such recombinant point mutation bearing viruses as observed in clinical trials as did recombinant viruses encoding for full-length, post-treatment envelope genes (Lu et al., 2004). Thus, T20-induced resistance development certainly has an effect on HIV-1 fitness as observed for other therapeutic strategies (Nijhuis et al., 2001). For further studies, pre-treatment samples from the same patient shall be used as reference envelopes (Simon et al., 2003) resulting in the most accurate assessment of fitness of HIV variants emerging over time as the result of effective T20 therapy.

Taken together, we have established a novel marker virus system that facilitates simultaneous assessment of viral fitness and entry inhibitor sensitivity using biologically relevant primary T-cells as targets. For T20-insensitive envelope proteins from treatment-naive patients, an inverse correlation between fusion inhibitor insensitivity and viral fitness was not observed. The efficient characterization of large numbers of Envs from pre- and post-treatment samples and quantitative analysis using multiple marker proteins will greatly enhance our understanding of the interplay between resistance and fitness. This system can also be used to compare escape from neutralizing antibody vs. fitness, which is important for understanding the consequences of neutralization escape.

Materials and methods

Plasmid construction

We previously described the plasmid clone pNL-ΔK (Dittmar et al., 2001) which was used in recombinant virus assays to determine coreceptor usage of primary envelopes cloned from HIV-positive individuals. To improve this system, the *nef* gene was replaced by one of three marker genes (Renilla luciferase, EGFP, or DsRed2) to create proviral derivatives that express one of the three reporter proteins (designated TN7Δ, TN6GΔ, and TN6RΔ, respectively). We chose renilla over firefly luciferase due to its smaller gene size. To facilitate directional cloning of patient-derived env genes, two unique restriction sites were introduced into these vectors. Both modifications, a *Bst*EII site (position 90 of the env gene) and an *Nco*I site (one nucleotide after the stop codon of the *env* gene, coinciding with the start codon of the marker genes) have been described previously (Polzer et al., 2001; Welker et al., 1998) (Fig. 1A). Here, we utilized the *Bst*EII–*Nco*I sites to clone and express different envelope genes amplified using PCR primers modified from Gao et al. (1998). Viruses expressing the well characterized NL4.3 (CXCR4-tropic, or X4) and Yu2 (CCR5-tropic, or R5) Envs were compared with those derived from three T20-naive individuals (R14, X10, and X23) (Derdeyn et al., 2001; Heil et al., 2004). Isolate X10 was previously shown to be the most T-20-insensitive virus from a panel of 55 primary isolates, and isolate X23 was the most insensitive to a related peptide inhibitor T-649, whereas R14 was unusual in its insensitivity to both inhibitors (Derdeyn et al., 2001). All *env* genes were cloned into each of the proviral clones.

Production and titration of virus stocks

To generate infectious virus stocks, 293T cells (1×10^6 cells) were transfected with 6 μg DNA and 15 μl Fugene6 (Roche, Germany) according to the manufacturer's instructions. Virus containing supernatant was harvested 3 days later and after filtration stored at −80 °C. For some experiments, the supernatant was concentrated 10-fold using Millipore filter devices to increase the titer. Virus stocks were titrated using the indicator cells TZM-bl which were seeded at a density of 3×10^3 cells per well in a 96-well tray. The next day, 5-fold dilutions of 293T-derived virus stocks were used to infect TZM-bl cells and 48 h later the cells were fixed with 3% paraformaldehyde and stained by the addition of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal; 0.5 mg/ml in phosphate-buffered saline (PBS) containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 1 mM magnesium chloride) (Wei et al., 2002). Individual groups of blue-stained cells were counted as single foci of infection and virus infectivity was determined as focus forming units. Titration on CD8-depleted PBMC (see below) was performed using a limiting dilution strategy with 5×10^4 cells per well in a 96-well tray and analyzed by a p24-ELISA 7 days post-infection.

Growth curve analysis and marker gene stability

Growth curve analysis was performed using either the T-cell line PM1, a subclone of HuT78 expressing CD4,

CXCR4, and CCR5 (Lusso et al., 1995), or CD8-T-cell-depleted PBMC. PBMCs from HIV-seronegative individuals were purified by the Ficoll-Hypaque method and depleted of CD8-positive T-cells using anti-CD8 magnetic bead-conjugated antibodies (Miltenyi, Germany). After stimulation with 0.5 μg/ml phytohemagglutinin A (PHA, Sigma, Germany) for 48 h, the cells were maintained in RPMI 1640 with 20% fetal calf serum (FCS), 20 U/ml recombinant human IL-2 (Roche, Germany), and antibiotics. Using equal amounts of infectious particles (TN7-NL, TN6G-NL and TN6R-NL), 1×10^6 stimulated donor PBMC or PM1 cells were infected (MOI of 0.001). After adsorption at 37 °C for 4 h, the cells were washed three times with PBS and observed for p24-CA production in the supernatant over time using an in house ELISA assay (Dittmar et al., 2001).

In order to evaluate the long-term stability of the marker gene, PM1 cells were infected successively at an MOI of 0.001 with each of the marker viruses expressing the NL4-3 envelope gene (TN7-NL, TN6G-NL and TN6R-NL) separately. Briefly, for the first passage, virus containing supernatant was harvested at 10 days post-infection and titrated on PM1 cells. A second passage was then initiated using the same MOI and virus harvested at day 10 post-infection. After the third consecutive passage, infected cells were fixed and permeabilized and the expression of EGFP and DsRed2 was evaluated in relation to intracellular p24 expression using PE- and FITC-conjugated anti-p24 antibodies (Coulter, Germany), respectively. In the case of TN7-NL, virus produced from the third passage was analyzed by comparing the expression of firefly luciferase induced in TZM-bl cells upon infection to expression of renilla luciferase due to virus infection (see Fig. 1C).

T20 inhibition studies with TZM-bl and PBMC

For the T20 inhibition studies on TZM-bl cells, equal amounts of infectious particles (1000 IU/well of TN7 derived infectious viruses) were added per well in the absence or presence of 10, 2, 0.4, 0.08, and 0.004 μg of T20/ml. Triplicate wells were analyzed for each drug concentration. For the T20 inhibition studies on PBMC, equal amounts of infectious particles (5000 IU/well of TN7 derived infectious viruses) were added per 1×10^5 PBMC in a round bottom 96-well tray in the absence or presence of 10, 2, 0.4, 0.08, and 0.004 μg of T20/ml. Each drug concentration was analyzed in triplicate. For both TZM-bl and PBMC, the cells were lysed at 2 days post-infection and the renilla luciferase activity (expressed due to infection with TN7 derived viruses) was measured using the renilla activity assay (Promega, Germany) and a luminometer (Labsystems, Germany). Relative activity (percentage of control) was calculated by dividing the mean number of relative light units at each T20 concentration by the mean number of relative light units from wells containing no drug.

Dual-color competition assay

The fitness of recombinant viruses carrying different *env* genes was determined by simultaneous infection with two viral species (e.g., TN6G-NL and TN6R-X10) at an MOI of 0.001 (determined using PBMC) in triplicate on 6-well plates. Each well contained 1×10^6 CD8-depleted PBMC in 1 ml RPMI medium. After 6-h adsorption at 37 °C, the cells were washed once in medium to remove residual input virus. To determine the relative replication capacity of one virus over the other, half of the cells were harvested 4 days post-infection, fixed with 3% paraformaldehyde, and analyzed by FACScan to measure the percentage of cells expressing EGFP (TN6G-derived viruses) or DsRed2 (TN6R-derived viruses) using the appropriate channels FL1 and FL2, respectively. The same analysis was performed with the remaining cells 8 days post-infection to confirm the first data set and to evaluate the growth advantage. All growth competition assays were performed at least three times independently in triplicate with PBMC isolated from different donors. Infection with two marker viruses expressing the same envelope (e.g., TN6G-NL and TN6R-NL) was included in each competition experiment for standardization. The results of all growth competition experiments were confirmed by expressing the opposite Env-reporter gene combination. The ratio of the number of EGFP-positive cells in channel FL1 vs. the number of DsRed2-positive cells in channel FL2 at day 4 and day 8 was measured to quantitate the growth difference for the respective recombinant virus pair. Similarly, the dual-color competition assay on PM1 cells was performed. PM1 cells were dually infected (MOI = 0.025 as determined using TZM-bl cells) with TN6G-NL and TN6R-GIA or the opposite virus pair. Cells were fixed 3 days post-infection and 7 days post-infection, and total EGFP fluorescence (508 nm) and DsRed2 fluorescence (582 nm) were determined using a multiwell fluorometer. The ratio of EGFP fluorescence vs. DsRed2 fluorescence correlates to the growth difference for the respective recombinant virus pair.

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