Journal of Biochemistry and Molecular Biology, Vol. 35, No. 3, May 2002, pp. 262-266

JOURNAL OF
Biochemistry and Molecular Biology

A Neutravidin-based Assay for Reverse Transcriptase Suitable for High Throughput Screening of Retroviral Activity

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Received 16 October 2001, Accepted 10 December 2001

A non-isotopic neutravidin-based reverse transcriptase (RT) assay adapted for high throughput screening of HIV activity is described. Using a 96-well microtitre plate, HIV particles are lysed and the RT enzyme released into a reaction mixture containing poly(A) RNA, biotinylated oligo d(T) and fluorescein-labelled dUTP (FI-dUTP). With poly(A) as a template and oligo d(T) as primer, the viron RT incorporates FI-dUTP into an elongating DNA strand. The resulting product is captured on a neutravidin-coated 96-well plate and the unincorporated nucleotides removed by a series of washing steps. A simple ELISA is subsequently performed using a monoclonal anti-fluorescein antibody conjugated to alkaline phosphatase. Quantification of RT activity is facilitated by a colorimetric readout. The assay was validated in the context of a diagnostic HIV-1 phenotyping assay. Using supernatants from HIV-1 infected lymphocyte cultures the assay was shown to be as sensitive as a radioactive assay and the RT activity correlated well with levels of cell-asociated HIV-p24. Importantly, even minor reductions of RT activity by virus variants with reduced fitness could be distinguished.

Keywords: HIV, RT-assay, Non-radioactive assay

Introduction

Diagnostic assays that allow the detection of human immunodeficiency virus (HIV) are today an integral part of drug development, antiretroviral treatment monitoring, and patient management (Hirsch *et al*., 2000). Currently, several methods are used for the routine analysis of HIV particles in patient plasma. Antigen capture enzyme-linked immunosorbent assay (ELISA) for quantification of the p24 gag protein of HIV-1 is commonly used for early diagnosis and to demonstrate virus replication (Lefrere *et al*., 1992). With the advent of HIV drug resistance testing for infected patients, recombinant DNA strategies were recently introduced which assess replication of resistant virus (Hertogs *et al*., 1998; Petropoulos *et al*., 2000). The direct detection of HIV-associated reverse transcriptase (RT), however, is also often used as a simple, accurate and broadly applicable assay system for the measurement of HIV replication, particularly in the context of *in vitro* assays (Corrigan *et al*., 1998).

The function of RT is to convert viral genomic RNA into a linear DNA duplex that is subsequently integrated into the viral genome (Tarrago-Litvak *et al*., 1994). The expression of viral proteins (including protease) and production of progeny virions are essentially linked to this process. Radioactive strategies have been widely implemented to detect HIV-associated RT activity. In these assays, ^{3}H- or ^{32}P- labelled thymidine triphosphate is incorporated into DNA in the presence of poly(A) template and an oligo d(T) primer. Environmental and safety issues, however, have led to the development and use of non-radioactive detection systems (Deaver, 1995). Nucleotides have been labelled with a variety of chemicals and the corresponding antibodies commercially produced for ELISA-based detection. Non-radioactive RT assays have been developed using various labels and detection systems (Ekstrand *et al*., 1996; Rytting *et al*., 1999).

Here, we describe a simple non-radioactive ELISA system suitable for the high throughput screening of HIV-1 associated RT activity using fluorescein as a label and commercially available neutravidin-coated plates. The assay is as sensitive as the corresponding radioactive assay and has been successfully linked to an HIV-1 phenotyping assay to detect viral drug resistance and to assess fitness of HIV variants in infection cultures. We have focussed here on HIV-1 associated activity. However this assay may be applied to the detection of RT activity of other retroviruses.

Materials and Methods

Neutravidin-based RT assay Supernatants from HIV-1 infected cells (final volume 10 µl) were lysed in 96-well plates containing

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50 µl Reaction Buffer (1 M Tris-HCl, 3 M KCl, 0.2 M DTT, 1 M $MgCl_2$, 0.5 M EDTA, 10% NP40, 10 µg polyA, 0.15 µg biotinylated oligo d(T), 0.1 nmol fluorescein-labelled dUTP (Roche Diagnostics, Germany)) per well and plates were subsequently incubated at 37°C for 1-2 h. Using a multichannel pipette, 220 µl Binding Buffer (TBS (25 mM Tris-HCl, 150 mM NaCl, pH = 7.2) containing 5 mM EDTA, 5 × Denhardts solution (0.25% Ficoll 400, 0.25% polyvinylpyrolidone, 0.5 µg/ml BSA) and 0.1% Tween 20) was added to each well and the samples transferred to a neutravidin-coated 96 well plate (Pierce, Rockford, IL, USA) prewashed in Wash Buffer (TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.2) containing 0.1% BSA and 0.1% Tween 20). Plates were incubated at room temperature for 60 min. Wells were washed three times with 200 µl TBS containing 0.3% Tween-20. Anti-fluorescein antibody conjugated to alkaline phosphatase (NEN, Boston, USA) was diluted 1 : 500 in TBS/0.3% Tween and 50 µl was added to each well. After a further 60 min incubation, wells were washed three times with TBS/0.3% Tween and 200 µl pNPP substrate (Pierce, Rockford, USA) was added. Plates were incubated until yellow colour could be measured colorimetrically at wavelength 405 nm (Titertek Multiscan Plus MKII, Labsystems, UK) typically after an overnight incubation step.

Radioactive RT assay The radioactive RT assay was performed as previously described (Klimkait *et al.*, 1998). In brief, the assay utilised ^{32}P-labelled dTTP (NEN) and oligo d(T) in a reaction mix that, after 2 h incubation at 37°C was dot blotted onto DEAE paper. The paper was washed three times and exposed on autoradiography film.

DNA transfection HIV genes isolated from the peripheral blood of HIV patients were cloned into an infectious HIV vector as described (Brennan LE, 2000). Approximately 0.2 µg cloned DNA was transfected into SX-22-1 HeLa cells (Klimkait *et al.*, 1998) using Lipofectamine 2000 according to the protocol supplied by the manufacturer (Life Technologies, Basel, CH). Cells were subsequently incubated in fresh complete DMEM-medium, containing HIV inhibitor at the indicated concentration, for 48 h prior to collection of cells and viral supernatants. For immunoblot analysis, cells were scraped from the plates and lysed as previously described (Klimkait *et al.*, 1998).

Lymphocyte infections Transfection supernatants were normalised for the number of viral particles present using transfection efficiency as a quantifiable marker (Klimkait *et al.*, 1998). For infection cultures, transfection supernatant was added to 1mL 8×10^4 CEM-SS cells in a 24-well plate. Infection supernatants were collected on days 4, 6, 8, 10, 12 and 15, centrifuged at 4000 rpm (Eppendorf tabletop centrifuge; Eppendorf, Hamburg, Germany) for 3 min prior to analysis by immunoblot or RT assay.

Results

Assay strategy In this assay, the reaction conditions optimised for HIV-associated RT activity was directly adapted from an established radioactive assay described above. Due to the availability of streptavidin- and more recently neutravidin-coated 96 well plates, we utilised this platform to capture synthesised DNA and therefore used biotinylated oligo d(T) primers. Fluorescein-labelled nucleotide was chosen due to its inherent properties that may, in the future, allow direct detection methods. All other assay conditions were optimised for sensitivity as indicated in Materials and Methods.

Assay reproducibility and sensitivity The HIV-associated RT activity of supernatants obtained from lymphocytes chronically infected with HIV-1 was determined using the neutravidin-based assay described. As indicated in Figure 1B, a series of supernatants (1×10^6 HIV RNA copies per µL) diluted by 10-fold steps in culture media defined the lower limit of detectable activity. These data were reproducible and the variability within three independent assays was not significant (Mean absorbance values for the 10, 3, 1 and 0,1 µL volumes were 0.11, 0.04, 0.03 and 0.01, respectively. The corresponding standard deviations were 0.012, 0.014, 0.0092 and 0.006, respectively).

Sensitivity and limit of detection of the assay were compared to those of a corresponding radioactive assay (Fig. 1A). Using both methods in parallel, we determined the RT activity in culture supernatants obtained as described above. Relative values and detection limits were consistently comparable between the two methods in three separate experiments.

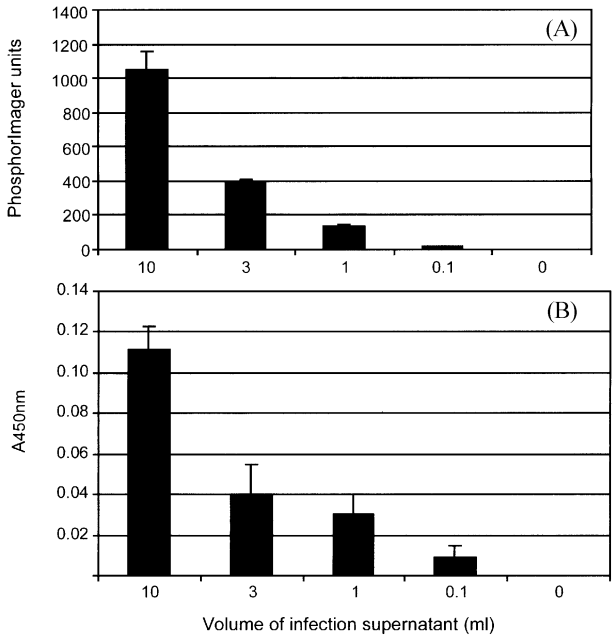

Fig. 1. Comparison of radioactive and neutravidin-based assays for HIV-associated RT activity. The indicated volumes of supernatant taken from HIV-1 infection cultures were analysed for HIV-associated RT activity using the radioactive assay (A) or the neutravidin-based non-radioactive assay (B). Intensity of radioactive signals was measured using a PhosphorImager and presented as PhosphorImager units. The neutravidin-based assay was analysed colorimetrically at wavelength 405 nm.

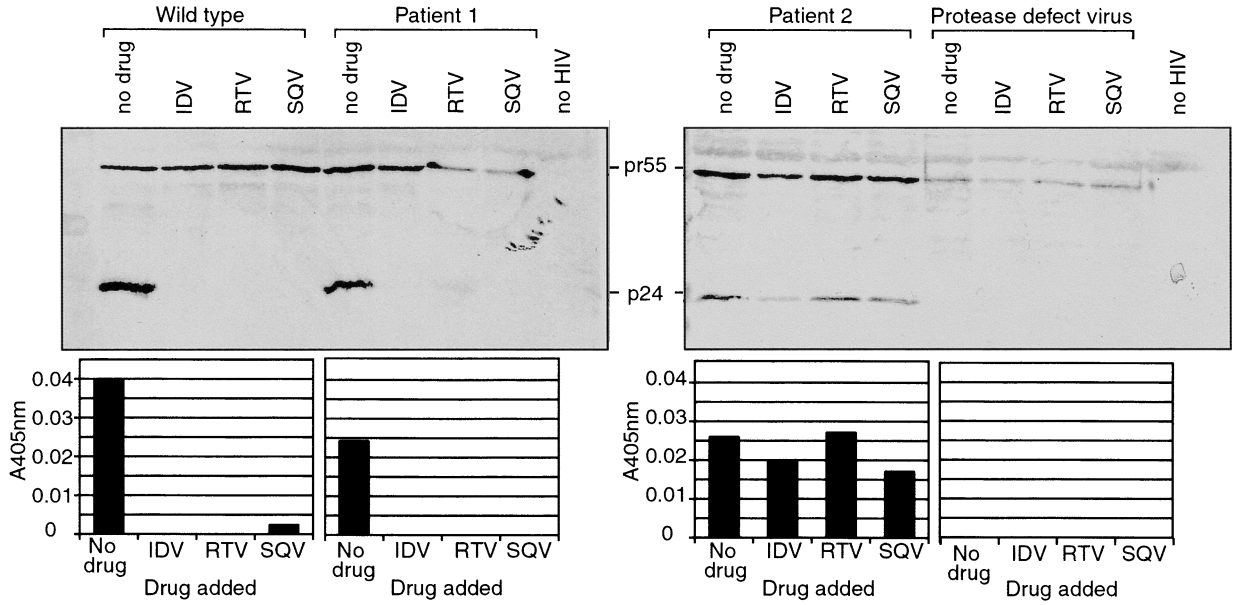

Fig. 2. The RT assay is used to determine HIV-1 activity in transfection supernatants containing HIV-1 protease inhibitors. Human HeLa cells were transfected with recombinant HIV-1 containing the protease genes isolated from the virus population of two patients. Similarly, both wild-type virus and that lacking a protease gene were analysed. Transfections were performed in the presence of 250 nM indinavir (IDV), saquinavir (SQV) and ritonavir (RTV) or in the absence of drug as shown. Untransfected cells (no HIV) were used as negative controls. Immunoblot analysis (upper) was as described and HIV pr55 and p24 proteins indicated. RT assay (lower) was performed as described in Materials and Methods.

Application to routine diagnosis of HIV drug resistance Recombinant HIV-1 variants, either resistant or sensitive to protease inhibitors (PIs), or wildtype virus were transfected into HeLa cells in the presence or absence of one of three PIs, ritonavir (RTV), saquinavir (SQV) and indinavir (IDV). After 2 days, we analysed supernatants for RT activity using our assay. Those viral variants resistant to PIs form mature particles and RT activity is detectable. Sensitivity to PIs allows inhibition of the protease therefore no viral particles form and RT activity is not detectable. It has been described that p24 is a reliable marker for HIV activity. As a control, we therefore utilised immunoblots to assess cell-associated HIV p24 levels.

As model examples, we examined the profiles of two recombinant HIV-1 variants derived from patient virus and compared them to that of the corresponding wild-type laboratory strain. As shown in Fig. 2, the detected RT activity correlated well with p24 formation. Wild type viral protease was sensitive to all inhibitors used and consequently the pr55 precursor could not be cleaved into the mature p24. Correspondingly, RT activity was also not detectable using the neutravidin-based assay. In patient 1 the respective HIV contained a protease that exhibited a wild-type immunoblot profile and, again, RT activity was not detected. Due to the sensitivity of the RT assay, however, it was possible to note that in the absence of drug, patient 1 produced less RT than wild-type (compare the OD of 0.04 with 0.025). In contrast, the HIV derived from patient 2 cleaved pr55 even in the presence of inhibitor and, in parallel, RT activity was readily detectable. As expected, the indicated variant lacking functional protease did not cleave pr55 or produce RT in the presence or absence of drug.

Application to routine diagnosis of HIV fitness Drug resistant variants of HIV-1 often possess a reduced replicative capacity (fitness) ex vivo, and it is becoming increasingly important for treatment strategies to assess the replication characteristics of these viruses in lymphocyte cultures. We have therefore tested the suitability of our RT assay as a tool to analyse the fitness of HIV variants in lymphocyte cultures. Supernatants taken from the transfections described above were normalised for transfection efficiency and subsequently added to lymphocyte cultures. Infection supernatants were collected over a series of days and the RT activity determined. Immunoblot assays were also performed as described above. As shown in Fig. 3, RT activity measurements accurately determine variations in the growth kinetics of HIV variants. Patient 2 derived HIV was demonstrated to possess reduced replicative capacity compared to that of both wild-type and patient 1. The viral activity indicated for patient 2 virus was not detected until Day 8 of infection compared to Day 6 for wild type infection. This RT assay can therefore be adapted to measuring the kinetics of HIV propagation in lymphocyte cultures.

Discussion

The further optimisation of anti-HIV-1 treatment regimens in the era of highly active antiretroviral treatment (HAART) has

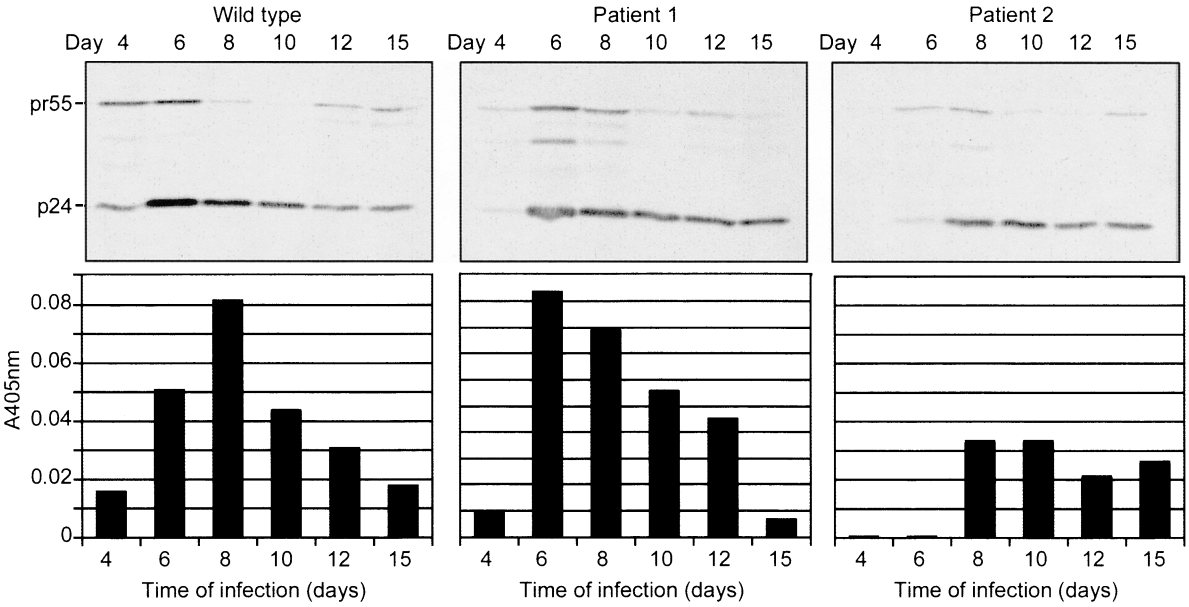

Fig. 3. The RT assay is used to determine levels of HIV-1 activity in infection cultures. Lymphocyte infections were prepared with transfection supernatants described in Fig. 2. Supernatants were collected at the time points indicated and analysed by immunoblot (upper) and RT assay (lower).

become possible through the introduction of assays that assess drug resistance profiles of a patient's HIV population (Durant *et al*., 1999; Perez-Elias *et al*., 2000). One such technology, phenotypic assays, utilise infection cultures to determine the replication capacity of HIV-1 isolates in the presence of drugs such as protease and reverse transcriptase inhibitors. High throughput assays were needed to evaluate HIV infections in vitro particularly for extended experiments and research studies (Richman, 2000). Previously, HIV-associated reverse transcriptase (RT) activity was often measured using radioactive methods that determine virion levels in infection supernatants. We have developed a non-isotopic, HIV-associated reverse transcriptase assay that can be adapted to high throughput screening of HIV activity. This assay provides a rapid and reliable method to detect variations in the kinetics and fitness of HIV in infections and has been successfully applied to HIV-1 phenotyping platforms.

Cost effectiveness and assay duration can limit the adaptation of assays to high throughput formats. Using the neutravidin-based system described here, cost can be reduced when compared to other non-radioactive assays formats such as the gag-ELISA. For high throughput analysis, the 96-well format is utilised, however, the availability of strip plates coated with neutravidin allows the adaptation of this assay also to smaller series which can further reduce cost. Commercially available precoated plates were incorporated into this assay for simplicity and reproducibility, but poly(A) coated plates have also previously been successfully adapted (McReynolds *et al*.,1999; Ekstrand *et al*., 1996). This ELISA-based system for RT analysis, in this format, is longer in duration than the corresponding radioactive assay. Nevertheless, when considering actual "hands on" time and the added convenience of omitting the need for radioactive steps, this system presents a favourable alternative. Further time reduction through adaptation of the enzymatic step may also be a possibility. Overall, the neutravidin-based assay can be simply adapted in all high throughput laboratories that require a flexible and reproducible method of detecting HIV-associated RT activity.

Acknowledgments This research was supported by grant number 32-53405.98 from the Swiss National Science Foundation.

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