FEBS Letters 447 (1999) 232–236 FEBS 21765

Eukaryotic expression of enzymatically active human immunodeficiency virus type 1 reverse transcriptase

Maria G. Isaguliants[a,b,*], Katja Pokrovskaya[c], Vladimir I. Kashuba[c,d], Dmitrii Pokholok[e], Jorma Hinkula[b], Britta Wahren[b], Sergey N. Kochetkov[e]

[a] *D.I. Ivanovsky Institute of Virology, Gamaleja str 16, 123098 Moscow, Russia*
[b] *Swedish Institute for Infectious Disease Control (SMI), Karolinska Institute, 171 82 Solna, Stockholm, Sweden*
[c] *Molecular and Tumourbiology Center (MTC), Karolinska Institute, 17177 Stockholm, Sweden*
[d] *Institute of Molecular Biology and Genetics, Zabolotnogo str 150, 252627 Kiev, Ukraine*
[e] *W.A. Engelgardt Institute of Molecular Biology, Vavilova str 32, 117984 Moscow, Russia*

Received 13 January 1999; received in revised form 29 January 1999

Abstract Reverse transcriptase of human immunodeficiency virus type 1 is a vitalenzyme in the HIV-1 replication cycle and an attractive target of attempts to arrest a primary viral infection. We designed a vector for eukaryotic expression of the 66 kDa subunit of reverse transcriptase under the control of the immediate early cytomegalovirus promoter. Efficient transient expression of the 66 kDa subunit of reverse transcriptase was achieved in a variety of cells. Immunostaining of the transfected cells revealed the cytoplasmatic localization of reverse transcriptase. Reverse transcriptase activity was detected in all transfected cell lines. Injection of this plasmid encoding the 66 kDa subunit of reverse transcriptase into mice resulted in strong reverse transcriptase-specific immune responses indicating that the 66 kDa subunit of reverse transcriptase is expressed in vivo. Sera from DNA-immunized mice inhibited reverse transcription in vitro.

Key words: Human immunodeficiency virus type 1; Reverse transcriptase; Eukaryotic expression; DNA immunization

1. Introduction

Reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) is a vital enzyme in the catalytic transformation of viral RNA into double-stranded linear DNA that is integrated into host cell chromosomes [1,2]. RT presents an attractive target in attempts to arrest the HIV-1 replication not only by means of chemotherapy. Antibodies to certain epitopes of RT inhibit its enzymatic activity [3,4]. A high prevalence of these antibodies was associated with asymptomatic infection and the inability to isolate virus from the sera of HIV-1 positive individuals [5,6]. A potent primary MHC class I-restricted CTL response was reported against functional domains of RT [7]. If an immune response directed against RT can interfere with the enzymatic activity and affect the virus viability, immunization against RT would have a clear protective potential.

To address this problem and establish the vaccine potential of RT, we constructed plasmid DNA for eukaryotic expression of the RT 66 kDA subunit (p66) that can form enzymatically active homodimers [8]. The efficient eukaryotic expression of enzymatically active p66 was achieved. The most advanced way of antigen delivery at the moment appears to be direct injection of antigen encoding DNA, i.e. genetic immunization [9–11]. In that case, antigens produced within the cell are correctly expressed, folded and glycosylated by the host. Our studies demonstrate that p66 encoding DNA directs the efficient expression of active enzyme in vivo and induces in immunized mice immune responses with a clear protective potential.

2. Materials and methods

2.1. Design of plasmid DNA encoding RT (pCMVRT)

The sequence encoding RT (HXB2) minus the His-tag and three C-terminal amino acids was excized from pBRT5 [12] and ligated into pSL1180 (Pharmacia, Sweden) at *Xho*I and *Xba*I sites of the polylinker in phase with the stop codon at the *Xba*I site. The resultant plasmid was partially digested with *Eco*RV to generate a fragment encoding RT with a restored stop codon flanked by *Eco*RV half sites (5′ terminal from pBRT5 and 3′ terminal from the pSL1180 polylinker). The fragment was cloned into a vector plasmid with the pUC8 backbone under the control of the cytomegalovirus immediate early promoter (CMV IE) promoter and the transcription termination signal from human papilloma virus type 16 [13] by cleaving the vector with *Sal*GI, filling in the cohesive ends with the Klenow fragment of DNA-polymerase, and digesting with *Hpa*I. The RT gene insert was confirmed by sequencing.

2.2. Expression of RT and preparation of cell lysates

Mouse fibroblasts NIH3T3, human Burkitt lymphoma cells DG 75 and monkey COS cells were transfected with pCMVRT by electroporation or with lipofectamin (Gibco BRL, UK). Samples were withdrawn after 24, 48 and 72 h and the expression of RT was analyzed by SDS-PAGE with subsequent Western blotting, indirect immunofluorescent microscopy (IFL), quantitative indirect ELISA and a RT activity assay. To assess the level of RT expression, cells were washed with PBS, collected by centrifugation, resuspended (10^6 cells/ml) in the lysis buffer containing 0.1 M Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 2 mM PMSF and 1 mM Trasylol (Sigma, USA) and kept on ice for 30 min. Nuclei were sedimented by centrifugation. Supernatant samples were diluted 1:1 (v/v) with ice-cold stabilizing buffer containing 0.1 M Tris-HCl, pH 8.3, 0.2 M NaCl, 10 mM dithiotreitol, 2% v/v Triton X-100 and 43.2% (v/v) glycerol and frozen at −70° to be further assessed for RT activity.

2.3. Preparation of anti-RT rabbit polyclonal IgG

RT of HIV-1 was expressed in *Escherichia coli* and purified by affinity chromatography [14]. Two rabbits were immunized by injections of RT at weeks 0, 2, 4 and 17. Injections on weeks 0 and 2 were done epidermal with 40 μg of RT in PBS in complete and subsequent

*Corresponding author. Fax: (46) (8) 33 72 72.
E-mail: maria.isaguliants@solna.mail.telia.com

Abbreviations: IFL, indirect immunofluorescent microscopy; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase of HIV-1; CMV IE, cytomegalovirus immediate early promoter

PII: S0014-5793(99)00297-5

boosts intravenously with 15 μg of RT in PBS in incomplete Freund adjuvant. Blood was taken 12 days after the last boost. RT-specific IgG was prepared by ammonium sulfate precipitation and dialysed against PBS. Control IgG was prepared from pre-immune rabbit sera.

2.4. RT detection and quantification

2.4.1. Western blotting. Western blotting was performed with RT-specific rabbit IgG and peroxidase-conjugated goat anti-rabbit immunoglobulins (DAKO) using a ECL detection kit (Amersham) according to the manufacturer's instructions.

2.4.2. IFL. Cells were seeded on cover slips 24 and 48 h post transfection and either fixed or grown for another 24 h. Suspension cells were cytospinned onto object glasses before fixation. Non-transfected cells and cells transfected with the vector plasmid [13] were prepared similarly. Immunostaining was done with either rabbit anti-RT IgG, or pre-immune rabbit IgG diluted in PBS containing 5% normal goat serum and 0.1% Triton X-100 as primary and swine anti-rabbit FITC-conjugated IgG (DAKO, Denmark) as secondary antibodies. Bisbenzimide (Hoechst 33258, 0.4 mg/ml) was added to the secondary antibody for DNA staining.

2.4.3. Indirect quantitative assay of RT. To prepare a calibration curve, known concentrations of RT (Boehringer Mannheim, Germany) were coated in duplicates on 96 well ELISA plates (MaxiSorb, Nunc, Denmark) in the concentration range 20 pg–200 ng per ml in carbonate-bicarbonate buffer, pH 9.6. Lysates of transfected and mock-transfected cells were coated in 2-fold dilutions up to 1:50. Free binding sites on the plate were blocked by incubation with PBS containing 5% BSA for 1 h at 37°. An indirect ELISA was performed as described earlier [15].

2.4.4. Assay of RT activity. Serial dilutions of the soluble fraction of lysates of pCMVRT-transfected cells were assayed using the non-radioactive RT kit (Boehringer Mannheim). The use of a substrate solution with enhancer allowed the detection as low as 1 pg of RT per well.

2.5. DNA Immunization

Three groups of C57B16 mice (eight each) were injected on days 1 and 26 with: (1) pCMVRT intra-muscularly (50 μg), (2) pCMVRT intra-epidermal (2 μg), (3) vector DNA with no insert intra-muscularly (50 μg). Blood was taken 9–14 days after each injection. Sera was analyzed by indirect ELISA. IFL was performed with immune and pre-immune sera on HIV-1_{LAI} infected and uninfected Jurkat tat cells using polyclonal rabbit IgG against HIV-1 gp160 as a positive and pre-immune mouse sera or PBS as negative controls.

2.6. Inhibition of reverse transcription by sera of DNA-immunized mice

Inhibition of reverse transcription by sera of DNA-immunized mice was performed using a non-radioactive RT assay kit (Boehringer Mannheim).

2.7. HIV-1 inhibition

HIV-1 inhibition was performed on HIV-1 laboratory strains LAI and SF2 with PHA-activated human peripheral blood lymphocytes as target cells. The inhibiting serum activity was analyzed at 3-fold dilutions starting from 1:10. Inhibition of infection by pre-incubation of HIV-1 or target cells with immune sera was measured by HIV-1 p24 determination [16]. Inhibition was defined as $>70\%$ reduction of the p24 viral antigen content in the supernatant as compared to the p24 content in experiments with pre-immune mouse sera.

3. Results and discussion

3.1. Eukaryotic expression of RT

The nucleotide sequence encoding RT of HIV-1 HXB2 [14] was inserted into an eukaryotic expression vector under the control of the CMV IE promoter to yield pCMVRT. Eukaryotic cell lines were transfected with pCMVRT and found to express a protein with a molecular mass of 66 kDa that reacted in Western blotting with rabbit anti-RT IgG where a strong band was seen (Fig. 1, lane 1). Only weak unspecific staining was registered for lysates of mock-transfected cells

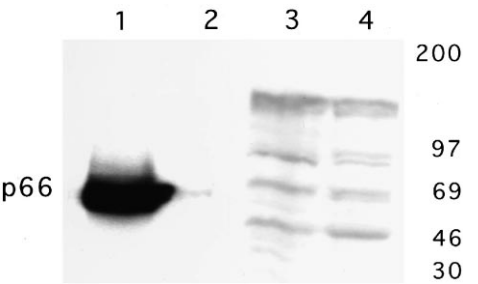

Fig. 1. Expression of RT subunit p66 in pCMVRT-transfected human DG75 cells. Cells were subjected to 15% SDS-PAGE and electroblotted onto a nitrocellulose membrane. Immunostaining of the Western blot was performed with rabbit anti-RT IgG, diluted 1:500 (lanes 1, 2) or pre-immune rabbit IgG, diluted 1:100 (lanes 3, 4). RT DNA-transfected cells (lanes 1, 3), mock-transfected cells (lanes 2, 4). Positions of the molecular mass markers are given to the right.

(Fig. 1, lane 2). Pre-immune rabbit IgG weakly stained lysates of both transfected and mock-transfected cells (Fig. 1, lanes 3 and 4, respectively).

RT expression was detected by IFL (Fig. 2A). Transfected cells showed no immunostaining with saline or pre-immune rabbit IgG (Fig. 2B). Anti-RT IgG did not recognize cells transfected with empty vector DNA (data not shown). Phase contrast image of the same fields demonstrated that RT was expressed preferentially in the cytoplasm.

The protein expressed was enzymatically active. RT activity was detected in the lysates of pCMVRT-transfected cells as the ability to extend the $(dT)_{15}$ primer on a poly(A) template. The enzymatic activity was registered only if the reaction mixture for reverse transcription contained less then 10 000 lysed cells. In control experiments, the addition of cell lysate from mock-transfected cells to recombinant RT led to a 50–90% inhibition of the RT activity. Inhibition of the RT activity by the components of a cell lysate is in concordance with results obtained previously by Lennerstrand et al. [17].

The amount of RT produced assessed by enzymatic activity was confirmed by quantitative indirect ELISA of cell lysates (data not shown) and estimated to be an average of 0.05–0.15 pg of RT per transfected cell, at 24 and 72 h post transfection, respectively. Thus, efficient eukaryotic expression of the enzymatically active p66 subunit of HIV-1 RT was achieved.

3.2. Immune response induced by the injection of pCMVRT

Expression of RT in pCMVRT-immunized mice lead to anti-RT antibody production in all animals, as was demonstrated by indirect IFL, Western blotting and ELISA (Fig. 3). In a number of pCMVRT-immunized mice, sera did not react with RT (group 2, A4: Fig. 3) but reacted with RT-derived synthetic peptides (data not shown). Sera from mice immunized with empty vector DNA did not react with RT in any of the immunoassays (see, for example, Fig. 3).

In mice injected intra-muscularly, anti-RT antibody titers ranged between 700 and 10 000, while in mice receiving intra-epidermal injections, the average titer was markedly lower reaching only 200. The latter might result from the lower dose of pCMVRT used, but could also reflect a difference in the route of delivery.

Sera collected after the second pCMVRT injection inhibited up to 70% of the RT activity in vitro (Fig. 4). Inhibition was exerted by the sera reactive with protein RT as well as by the

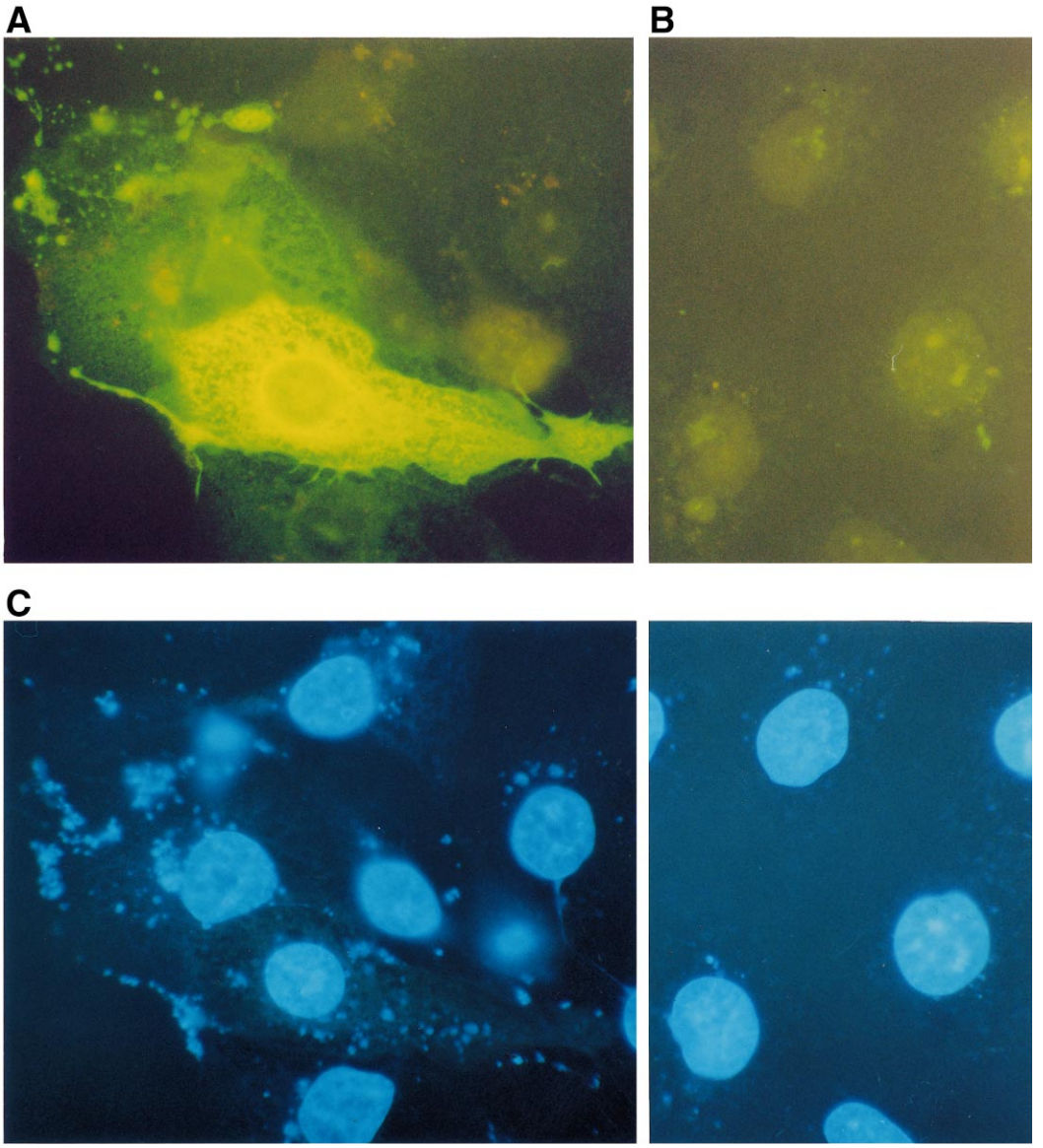

Fig. 2. Detection of RT by IFL of pCMVRT-transfected COS cells. The upper panel shows two fields after staining with rabbit anti-RT IgG (A) or pre-imune rabbit IgG (B) and FITC-conjugated swine anti-rabbit IgG. The lower panel shows nuclei staining of cells in the same fields (C).

sera reacting with RT-derived peptides only (group 1, A2, B1 and group 2, A4, respectively, Fig. 4). The effect observed in the presence of normal mouse sera was less than 20% inhibition at a 1:10 dilution (data not shown). Sera from mice immunized with vector DNA had a certain inhibitory activity, however the latter was markedly lower than the inhibitory effect of the sera from pCMVRT-immunized animals (group 3, Fig. 4).

The inhibition of the RT activity by the sera of pCMVRT-immunized mice could result from induction of antibodies against regions of RT involved in the enzymatic activity. Reactive B-cell epitopes were previously localized in the RT regions involved in binding of template and template primer, catalysis of polymerization, translocation of the template primer following nucleoside incorporation and RNase H interaction with DNA [3,4,18].

The nature of the low inhibitory effect exhibited by the sera of empty vector DNA-immunized mice is unclear. One of the possible explanations could be induction in the course of DNA immunization of anti-nucleic acid antibodies [19]. Anti-nucleic acid antibodies could bind primer and/or template and thus prevent the efficient reverse transcription. This possibility has to be further investigated.

Our preliminary experiments demonstrated that sera from pCMVRT-immunized mice inhibit replication of laboratory strains of HIV-1 SF2 and LAI (data not shown). Sera of mice immunized with empty vector DNA also exhibited a low inhibitory activity if target cells were pre-incubated with sera prior to HIV-1 challenge. For anti-RT antibodies, virus inhibition/neutralization might be a result of the ability of antibodies to enter cells, inhibit the RT activity (as was demonstrated in the in vitro experiments) and thus interfere with the HIV-1 replication cycle. Intracellular neutralization of virus by immunoglobulin A antibodies, that are able to form complexes with newly synthesized viral proteins and thus prevent HIV-1 transmission, was described [20]. The mechanism

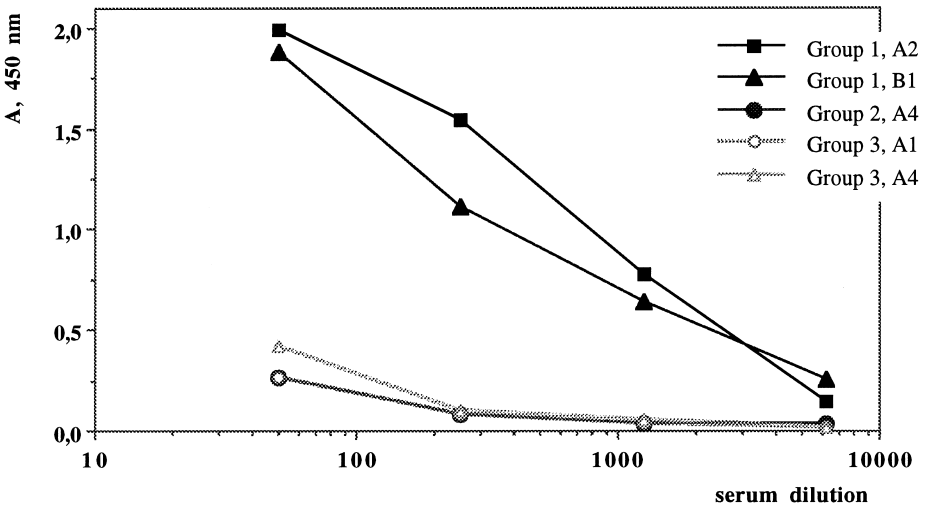

Fig. 3. Titration of anti-RT antibodies in sera of mice immunized with pCMVRT intra-muscularly (group 1, mice A2, B1), intra-epidermally (group 2, A4) and with vector DNA containing no gene insert intra-muscularly (group 3, A1, A4).

of HIV-1 inhibition/neutralization by antibodies induced in DNA immunization has yet to be understood.

The next step will be assessing the ability of the immune response against RT expressed in situ to protect mice against a challenge with HIV-1 pseudotype (amphotropic murine leukemia virus/HIV-1 with expanded cellular and species tropism, [21]). Preliminary experiments demonstrated an up to 60% protection in mice receiving intra-muscular pCMVRT injections, while no protection was conferred to empty vector DNA-immunized mice.

Acknowledgements: This work was supported by Grants of Swedish Royal Academy of Science #1351, #12676 and of the International Association for the promotion of cooperation with scientists from the New Independent States of the former Soviet Union #96-1565.

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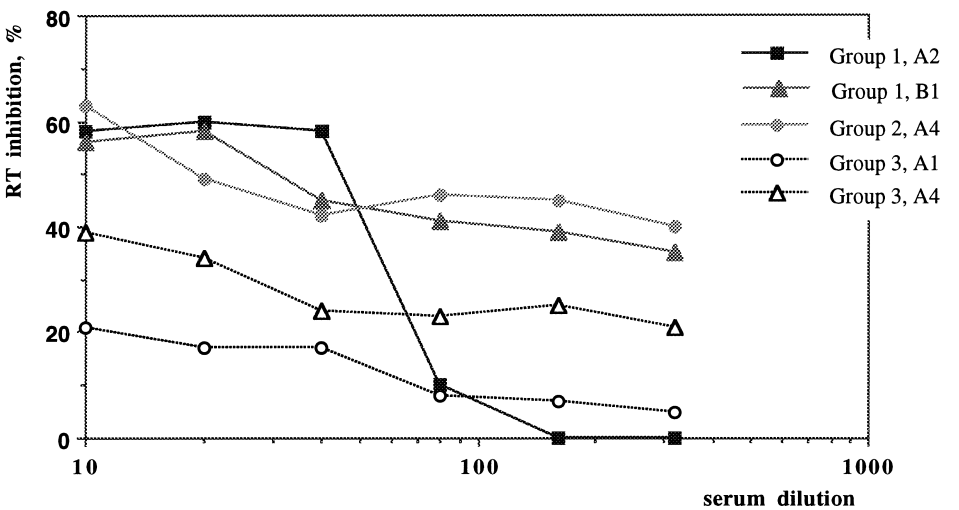

Fig. 4. Inhibition of RT activity by sera of mice immunized with pCMVRT intra-muscularly (group 1, mice A2, B1), intra-epidermally (group 2, A4) and with vector DNA containing no gene insert intra-muscularly (group 3, A1, A4). The data are expressed as relative percentage of inhibition compared with the pre-immune mouse serum at a dilution of 640.

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