Inhibition of murine leukaemia virus retrotranscription by the intracellular expression of a phage-derived anti-reverse transcriptase antibody fragment

Nicola Gargano and Antonino Cattaneo
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

The intracellular targeting of recombinant antibodies is an experimental strategy to interfere with the function of selected molecules that is being utilized in a variety of different systems for research and medical applications. Since recombinant antibodies are increasingly being derived from phage display libraries, we have exploited phage technology to isolate, from a large combinatorial library, human antibody fragments directed against human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT). We describe in this paper the in vitro and in vivo properties of a neutralizing anti-RT antibody fragment. We demonstrate that the heavy chain domain (VH-CH1) of the phage-derived antibody is able to inhibit the retroviral enzyme, in that it neutralizes the RNA-dependent DNA polymerase activity of HIV-1 RT. The VH-CH1 antibody fragment also neutralizes the activity of RT of drug-resistant HIV-1 mutants as well as that of murine retrovirus RT. To confirm the broad reactivity of the synthetic antibody fragment, we have assessed the ability of the intracellularly expressed VH-CH1 protein to interfere with murine retroviral infection. To this end, we developed an in vivo selection procedure based on the antibody-mediated resistance to a cytotoxic retrovirus and used this selection procedure to rescue, from a heterogeneous population, cells expressing the VH-CH1 antibody fragment. We finally demonstrate that the intracellular expression of the recombinant heavy chain antibody fragment leads to an efficient inhibition of viral retrotranscription by murine-based retrovirus.

Introduction

The intracellular expression of recombinant antibodies targeted to different intracellular compartments (Biocca & Cattaneo, 1995; Biocca et al., 1990; Cattaneo & Biocca, 1997) is an experimental strategy aimed at interfering with the function of selected molecules, that is being increasingly utilized in a variety of different systems (Richardson & Marasco, 1995; Tavladoraki et al., 1993). This experimental approach has been successfully applied to confer a certain degree of protection against retroviral infection and replication, by expressing intracellular antibody fragments against a variety of viral proteins (Duan et al., 1994; Maciejeski et al., 1995; Marasco et al., 1993; Mhashilkar et al., 1995; Shaheen et al., 1996). Recombinant antibodies are increasingly being derived from phage display libraries, but no phage-derived antibody has been expressed intracellularly in mammalian cells. We have recently exploited phage technology to isolate, from a large synthetic library of human Fab antibody fragments (Griffiths et al., 1994), anti-human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) antibodies suitable for intracellular expression. We have recently described the isolation and characterization of two antibody fragments that completely neutralize the RNA-dependent DNA polymerase activity of HIV-1 RT at nanomolar concentrations, as well as that of RTs from different classes of retrovirus (Gargano et al., 1996). In this paper, we provide a further characterization of one of the selected antibody fragments (D7) (Gargano et al., 1996), by studying the relative contribution of the two isolated chain domains (VH-CH1 and VL-CK) of the phage-derived Fab fragment to the inhibition of the retroviral enzyme. We show here that the heavy chain domain of the synthetic phage-antibody (VH-CH1 D7αRT), whilst retaining the ability to neutralize the RNA-dependent DNA polymerase activity of HIV-1 RT, also inhibits the activity of drug-resistant HIV-1 RT mutants, as well as that of murine retroviral RT. To gain insight into the broad spectrum of reactivity of the VH-CH1 anti-RT antibody fragment, we assayed in vivo the inhibition of RT by challenging murine fibroblast cells expressing the synthetic antibody fragment with Moloney murine recombinant retrovirus. We developed an in vivo selection procedure, based on
the antibody-mediated resistance to the toxic action of a retroviral encoded gene and used this selection procedure to rescue and expand cells intracellularly expressing the neutralizing VH-CH1 D7αRT antibody fragment. Finally, we demonstrated a tight correlation between inhibition of viral retrotranscription and expression of the phage-derived VH-CH1 D7αRT antibody fragment. This successful inhibition of viral retrotranscription by the cytosolic expression of the synthetic antibody fragment presents the prospect of using phage display technology as a new source of recombinant antibodies suitable for intracellular immunization schemes.

Methods

**Construction of VH-CH1 expression vectors.** The heavy chain antibody fragments VH-CH1 D7αRT and VH-CH1 αFITC were derived respectively from Fab antibody fragment D7 (Gargano et al., 1996) and from Fab αFITC-BO2 (Griffiths et al., 1994). Plasmid pUC119-mycpolyHis, kindly provided by A. Griffiths, was used as bacterial expression vector, while plasmid pScFvexp (Persic et al., 1997) was used for the cytosolic expression of antibody fragments in mammalian cells. Plasmids pUC119-VHCH1-D7αRT and pUC119-VHCH1-BO2αFITC were constructed by subcloning NcoI–NolI fragments from pUC119-FabD7 and pUC119-FabBO2αFITC respectively into NcoI–NolI cut pUC119-mycpolyHis, with standard techniques (Sambrook et al., 1989). For expression in mammalian cells, plasmids pScFv-VHCH1-D7αRT and pScFv-VHCH1-BO2αFITC were constructed by subcloning NcoI–NolI fragments from pUC119-FabD7 and pUC119-FabBO2αFITC respectively into NcoI–NolI cut pScFvexp. These vectors direct the expression of cytosolic antibody fragments under the transcriptional control of the EF-BOS (elongation factor-1a) promoter (Persic et al., 1997).

**Characterization of heavy chain fragment and enzymatic assays.** For bacterial expression, TG1 cells were transformed with pUC119-VHCH1-D7αRT and pUC119-VHCH1-BO2αFITC plasmids and soluble expression of heavy chain antibody fragments was induced with IPTG as previously described (Marks et al., 1991). VH-CH1 antibody fragments were purified via their histidine tag on Ni–NTA resin (Qiagen) from the periplasmic fraction, according to the manufacturer's instructions. After elution from the column with imidazole elution buffer (50 mM sodium phosphate buffer, pH 7.5, 500 mM NaCl, 100 mM imidazole), the protein was concentrated (Centricon 10) and dialysed against PBS.

RT RNA-dependent DNA polymerase activity was measured in an assay consisting of 10 nM recombinant HIV-1 RT (Gargano et al., 1996) in RB buffer (RB = PBS, 2.5 mM MgCl₂, 15 μM [α-32P]dGTP, 10 μM dCTP, 10 μg/ml poly(rC)/oligo(dG), 0.1 mg/ml BSA), in a total volume of 20 μl. After incubation for 30 min at 37 °C, the reaction was terminated by addition of 1 ml of ice-cold PBS and the mix was filtered on DE81 chromatographic paper, followed by washing of the filters three times with 5% NaHPO₄ and once with water. Radioactivity bound to the filters was finally counted. The activity of heavy chain antibody fragments was monitored by preincubation of VH-CH1 purified proteins with HIV-1 RT in RB buffer for 15 min on ice, then [α-32P]dGTP and poly(rC)/oligo(dG) were added and the reaction was started as above. The assay for other viral RT enzymes was performed under identical conditions.

HIV-1 LTR [dideoxyinosine (ddI) resistant] and HIV-1 RTMC [dideoxynucleoside (AZT) resistant] recombinant RTs were kindly provided by Dave Stammers. Moloney murine leukemia virus (MMLV) RT was purchased from Promega, as an RNase-H deficient enzyme.

**Cell cultures and viruses.** Murine fibroblast BALB/c-3T3 cells were cultured in DMEM medium supplemented with 10% foetal calf serum (FCS). Transfection of BALB/c-3T3 cells with the cytosolic expression vectors pScFv-VHCH1-D7αRT and pScFv-VHCH1-BO2αFITC was performed by electroporation: cells were trypsin harvested, washed twice with DMEM and resuspended in 0.3 ml DMEM (1.3 x 10⁵ cells/ml) to which 10 μg of pScFv plasmid DNA was added. The mixture was incubated for 10 min on ice and electroporated in 0.4 cm cuvettes with one pulse of 0.4 kV/500 μF. The cells were then maintained for 10 min on ice, resuspended in 10 ml DMEM–10% FCS and incubated for 24 h at 37 °C. Uncloned transfected cells were collected after 2 weeks of neomycin (G418; 1 mg/ml) selection in DMEM–10% FCS.

The B16-PAGO ecotropic retrovirus packaging cell line, kindly provided by R. Vile, was used as a source of MMLV-PAGO replication-defective viruses (Vile et al., 1994). This retrovirus encodes the expression of the herpes simplex virus thymidine kinase (HSV-1 tk) gene, which confers to cells sensitivity to the toxic action of gancyclovir (GCV). The Mo-LacZ ecotropic packaging cell line TELCeB6 (Cosset et al., 1995), kindly provided by S. Russell, was used for the generation of MMLV-LacZ replication-defective viruses. Both packaging cell lines were grown in DMEM–10% FCS supplemented with penicillin–streptomycin (100 μg/ml) and, for the B16-PAGO cells, with neomycin (2.5 mg/ml).

*Autographa californica* multiple nucleopolyhedrovirus strain E2 (AcMNPV) and the baculovirus expression vector pVL1393 were from Pharmingen. Recombinant baculovirus harbouring the VH-CH1 D7αRT antibody-fragment-encoding sequence (BV-VH-CH1-D7αRT) was constructed by cotransfection of plasmids pVL1393-VHCH1-D7αRT with AcMNPV DNA into Sf9 cells. Plasmid pVL1393-VHCH1-D7αRT was constructed by subcloning PnuI–XhoI fragments from pScFv-VHCH1-D7αRT into PnuI–XhoI cut pVL1393. Recombinant baculoviruses encoding the VCH1-D7αRT antibody fragment were isolated by plaque purification, as previously described (Summers & Smith, 1987). *Spodoptera frugiperda* cells (Sf9) were grown at 27 °C in TNM-FH medium (Sigma), supplemented with 10% heat-inactivated FCS.

**β-Galactosidase assays of MMLV-LacZ-infected cells.** For transduction of antibody fragment BALB/c-3T3-expressing cells with MMLV-LacZ replication-defective viruses, 2 ml of supernatant of subconfluent TELCeB6 packaging cells (16 h production of MMLV-LacZ virus; titre 5 x 10⁵ c.f.u./ml) was used to infect 10³ target cells by incubation with Polybrene (4 μg/ml) for 5 h at 37 °C. After infection, the medium was replaced and the transfected cells were maintained in culture for 3 days. The β-galactosidase enzymatic activity of the virally encoded reporter protein was then detected (a) qualitatively in fixed cells or (b) quantitatively in cell extracts.

(a) β-Galactosidase assay in fixed cells. Transfected cells were washed twice with PBS and fixed for 10 min with 4% paraformaldehyde in PBS. Fixed cells were incubated for 5 min in rinsing solution (0.1% BSA, 0.25% Triton X-100 in PBS) and then washed for 5 min in X-Gal buffer (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂ in PBS). Finally, β-galactosidase expression in transfected cells was revealed by incubation in stain solution (50 μg/ml 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside in X-Gal buffer) for 1 h at 37 °C and the reaction was stopped by washing the cells with PBS.

(b) Quantitative β-galactosidase assay. Infected cells were washed twice with PBS, lysed in 0.2 ml lysis buffer (0.1% SDS in PBS) for 10 min on ice and 0.8 ml of ice-cold PM buffer (33 mM NaH₂PO₄, 66 mM Na₂HPO₄, 0.1 mM MnCl₂, 2 mM MgSO₄, 40 mM 2-mercaptoethanol) was then added to fully resuspend the lysates. Cell extract (20 μl) was mixed with 0.5 ml PM-2 buffer (0.8 mg/ml o-nitrophenyl β-d-galactopyranoside in PM buffer) and the samples were incubated at 37 °C to start the enzymatic reaction. Reactions were stopped at different times...
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**Fig. 1.** Characterization of VH-CH1 D7αRT. (A) Western analysis of affinity-purified VH-CH1 D7αRT (lanes 1, 4), VH-CH1 BO2αFITC (lanes 2, 5) and ScFv αNGF (Ruberti et al., 1993) (lanes 3, 6) proteins expressed by placing the samples on ice and adding 0.25 ml 1 M Na₂CO₃. Finally, the samples were briefly frozen, thawed, centrifuged at 12000 g and the A₅₉₅ was measured for each supernatant.

**PAGO challenge.** For infection of fibroblast BALB/c-3T3 cells with MMLV-PAGO replication-defective virus, a total of 10⁶ cells was serially incubated with 7 ml of supernatant of subconfluent B16-PAGO packaging cells (12 h production of MMLV-PAGO virus; titre 10⁶ c.f.u./ml) as follows: (a) infection for 5 h with Polybrene (4 µg/ml); (b) infection for 12 h; (c) infection for 5 h with Polybrene (4 µg/ml); (d) infection for 12 h. After infection, the cells were maintained for 12 h in growth medium and then incubated in the presence of GCV (10 µg/ml) with no other selection for 3 weeks, changing the medium every 3 days. To examine the expression level of antibody fragments in the infected cell population, at different times of GCV selection cell samples were washed twice with PBS and solubilized in SDS-sample buffer. After separation on 15% SDS–PAGE, proteins were transferred to nitrocellulose membranes and immunoblotted with the monoclonal antibody MAb-9E10 (1 µg/ml), recognizing the C-terminal Myc-tag. Blots were treated with horseradish peroxidase-labelled anti-mouse IgG and visualized by using the ECL system (Amersham).

**Inhibition of viral retrotranscription.** Since the MMLV-LacZ retrovirus is replication-defective, the cellular levels of viral DNA were monitored in a ‘one-step infection’ assay. For this purpose, BALB/c-3T3 cells expressing the recombinant antibody fragments were challenged with MMLV-LacZ virus as described above, and 3 days after infection the amount of viral DNA was measured. Total cellular DNA was prepared by a quick lysis method. In brief, 10⁶ cells were solubilized in 100 µl of lysis buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.5% NP40, 0.5% Tween 20, 50 µg/ml proteinase K) and incubated for 1 h at 56 °C. The samples were then boiled for 10 min to inactivate the protease K. The amount of viral DNA was calculated by semi-quantitative PCR analysis using specific primers for the lacZ reporter gene (5’ primer, 5’ CCATGATACCGGATTCACTGCGCGT 3’; 3’ primer, 5’ GCAACGGCTTGCCGTTCAGCAGCAG 3’). To normalize for cellular DNA, mouse α-tubulin DNA was amplified with 10-fold diluted samples (5’ primer, 5’ GCTTTGACTCATATGGCCATGGC 3’; 3’ primer, 5’ TCAGGGATCCGGGATTCTGACGAGATGTC 3’). PCR products were then separated on 1.5% agarose gels, transferred onto Hybond-N+ membranes, hybridized with 5’-end-labelled ³²P-labelled oligonucleotide probes (T4 kinase reaction) and revealed by autoradiography. The hybridized products were quantified with a PhosphorImager (Molecular Dynamics). The DNA standard curves used for quantification were derived by titrating the PCR DNA input from plasmid pMFG-nlsLacZ (kind gift of S. Russell). The levels of viral DNA (copy number) were calculated on the basis of the DNA standard curves, and the percentage changes compared with control
values were calculated on the basis of quantitative measurements of hybridized products. A subsaturating number of PCR cycles, as determined from preliminary experiments, was performed (20 and 22 cycles were routinely performed), both for the calibration curves and for the cellular DNA samples.

**Results**

**Neutralization of RTs of diverse origin by a synthetic phage-derived human antibody fragment**

The broad reactivity of the antibody fragments previously isolated from a large combinatorial library of human Fab antibody fragments (Gargano et al., 1996) suggested their potential use to inactivate retroviral RT by intracellular expression, in a gene therapy perspective. To gain insight into the neutralization properties of the synthetic anti-RT antibody fragments (Gargano et al., 1996), we performed experiments to evaluate the relative contribution of the heavy and light chains of the phage-derived Fab antibody fragment D7αRT to the inhibition of the retroviral enzyme. Sequencing of DNA encoding for the antibody domains showed that in all the neutralizing phage-derived antibody fragments previously studied (Gargano et al., 1996) the heavy chain variable domains belong to an identical germ-line VHα subfamily, whilst having different light chain variable domains (data not shown). We therefore engineered the expression of the heavy chain alone (VH-CH1 fragment) of D7αRT in *E. coli*. The resulting protein was purified from the periplasmic space by affinity chromatography and the Western analysis is shown in Fig. 1(A). Under non-reducing conditions, the majority of the VH-CH1 protein runs as a doublet, slightly faster than a single chain Fv fragment. The doublet most likely reflects a heterogeneity in the formation of the intrachain disulfide bonds, with the higher band corresponding to the species with reduced disulfide bonds. RT enzymatic assays were performed with this protein preparation, and the results showed that the heavy chain alone (VH-CH1 D7αRT) neutralizes the RNA-dependent DNA polymerase activity of HIV-1 RT (Fig. 1). The VH-CH1 D7αRT antibody fragment displays 50% neutralization under conditions of 50 nM antibody concentration, as shown in Fig. 1(B), where a wider range of antibody concentrations was explored. A control heavy chain antibody fragment derived from the phage-antibody Fab-BO2 anti-FITC (Griffiths et al., 1994), expressed and purified under identical conditions, induced no inhibition of RT activity. The inhibition of RT activity by the VH-CH1 D7αRT antibody fragment is competitive with respect to the RNA substrate, as demonstrated in Fig. 1(C), where the results are reported in the form of a double reciprocal plot. To further characterize the inhibitory properties of VH-CH1 D7αRT protein, we tested its ability to interfere *in vitro* with the activity of HIV-1 drug-resistant RT mutants (Leu14–Val ddl resistant; RTMC, AZT resistant), as well as that of RT from murine retrovirus. The results in Fig. 1(D) show that the RTs of the two HIV-1 drug-resistant mutants are similarly neutralized by VH-CH1 D7αRT. Also, the RT activity from the murine isolates is neutralized by the phage-derived VH-CH1 D7αRT, albeit less effectively (around 60% inhibition with 100 nM D7αRT). This is noteworthy in view of the fact that human and murine RT enzymes have a high conserved structural homology in the catalytic palm domain motifs (Georgiadis et al., 1995), thus indicating that a common epitope is recognized by the phage-derived VH-CH1 D7αRT protein.

**Intracellular expression of VH-CH1 D7αRT antibody fragment**

The availability of an active heavy chain fragment, as opposed to a complete Fab fragment, would facilitate intracellular expression, since it requires the expression of only one subunit of the recombinant antibody. Therefore, a vector for the cytosolic expression of the VH-CH1 D7αRT protein in mammalian cells was constructed (Fig. 2A), in which the leader sequence for antibody secretion was removed (leaderless). To assess the ability of the intracellularly expressed phage-derived antibody fragment to inhibit viral retrotranscription, mammalian cells expressing the VH-CH1 D7αRT antibody fragment were generated. Cells expressing a control VH-CH1

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**Fig. 2.** Eukaryotic constructs. (A) Schematic representation of antibody fragments used for intracellular expression in murine BALB/c-3T3 cells. EF-BOS, elongation factor 1α promoter; VH, variable heavy chain region (VHα family subclasses); CH1, human IgG1 heavy chain constant region; myc, tag epitope recognized by MAb 9E10. (B) Replication-defective retroviral vectors used for infection of BALB/c-3T3 cells. LTR, MMLV long terminal repeat; Ψ, viral RNA packaging signal; TK, herpes simplex virus thymidine kinase; LacZ, *E. coli* β-galactosidase; NLS, nuclear localization signal; neo, neomycin; fleo, fleomycin.
protein (from the Fab-B02αFITC) were also derived. The intracellular distribution of the VH-CH1 D7αRT protein, as judged by immunofluorescence, was typical of a leaderless antibody fragment targeted to the cytosol (Biocca & Cattaneo, 1995) (not shown). To assess the broad reactivity shown by the phage-derived antibody fragment, mouse fibroblast BALB/c-3T3 cells expressing the VH-CH1 D7αRT antibody fragment were challenged with murine-based retrovirus (derived from MMLV). Two replication-defective recombinant viruses were used, MMLV-LacZ (Cosset et al., 1995) and MMLV-PAGO (Vile et al., 1994), respectively encoding a reporter gene for *E. coli* β-galactosidase (harbouring a nuclear localization sequence) and HSV-tk. These defective retroviruses, schematically shown in Fig. 2(B), were used for one-step infection of BALB/c-3T3 transfectants, without further production of retroviral particles. Uncloned cell transfectants were utilized for virus challenge experiments, rather than stable clones chosen on the basis of high expression, as this would allow a virus-dependent selective pressure to be imposed on cells intracellularly expressing the neutralizing antibody fragments.

BALB/c-3T3 cells expressing either the VH-CH1 D7αRT protein or an irrelevant antibody fragment (VH-CH1 B02αFITC) were infected with MMLV-LacZ and the enzymatic activity of the virally encoded β-galactosidase was determined 72 h later by *in situ* staining. Infection was carried out at high m.o.i. (in the order of 2x10^3 c.f.u. per cell) in order to allow detection of variations based on the number of integrated
proviruses. Fig. 3 shows staining of MMLV-LacZ infected cells for β-galactosidase, control transfectants in (A) and VH-CH1 D7αRT-expressing cells in (B). In the latter case there is a marked overall reduction of the intensity of β-galactosidase nuclear staining, indicating lower expression of the viral reporter gene in VH-CH1 D7αRT-transfected cells. The staining intensity of VH-CH1 D7αRT-transfected cells is heterogeneous, probably reflecting the heterogeneity found for the expression of the antibody fragment in the uncloned transfected population. The conditions of high m.o.i. allowed detection of a titration effect by the intracellular antibody fragments. This was confirmed by experimental determination of the number of integrated proviruses (see below, Fig. 5).

**Using virus killing as a selective pressure for intracellular antibody fragments**

The use of lacZ as a reporter gene, while providing a very convenient assay to monitor the expression of viral genes, does not allow imposition of a virus-dependent selective pressure on the infected cells. This would be desirable, in order to study the effectiveness of intracellular antibody expression in conferring resistance to the action of a cytotoxic retrovirus. To this aim, we used the PAGO retrovirus (Vile et al., 1994), an MMLV-based defective retrovirus in which the HSV-tk gene is expressed under the control of the viral LTR. Expression of the HSV-tk gene converts the antiviral compound GCV into a toxic form, thus leading to GCV-dependent cell death. BALB/c-3T3 cell transfectants expressing VH-CH1 D7αRT and the control VH-CH1 B02xFITC antibody fragments were infected with PAGO retrovirus, and GCV was added 24 h later. The susceptibility of cell transfectants to the virus-dependent toxic effect of GCV was assessed by counting the surviving cells as a function of time. Fig. 4(A) shows that both the cultures show extensive cell death during the first week, with a greater number of surviving cells in the VH-CH1 D7αRT transfectants. In the following days the difference with control cultures expressing the irrelevant heavy chain antibody is amplified, as the former cells grow exponentially, while the latter continue to die. These results demonstrate that intracellular expression of the VH-CH1 D7αRT antibody fragment leads to protection of the cells from the toxic effects resulting from PAGO retrovirus infection. The initial phase of cell death reflects the non-clonal nature of the transfected population, and the following growth phase represents the expansion of the resistant cells in the initial population.

In order to verify whether resistance to the toxic effect of the virally expressed HSV-tk is related to expression of the antibody fragment, the amount of VH-CH1 D7αRT protein in the cell population was monitored at different times following addition of GCV. Western analysis (Fig. 4B) shows that the band corresponding to the VH-CH1 protein is not visible in the unselected polyclonal population (lane 1), becoming visible after 4 days of GCV selection (lane 2), and reaching the maximum level after 2 weeks of GCV selection (lane 3). This experiment allowed us to conclude that resistance to GCV correlates with expression of the VH-CH1 D7αRT antibody fragment in the cell population, thus indicating that exposure of the uncloned cell population to the toxic action that results from virus infection allows selection of cells intracellularly expressing the neutralizing anti-RT antibody fragment from the initial mixed population.

**Inhibition of viral retrotranscription by the phage-derived antibody fragment**

In order to provide further support for the correlation between expression of the phage-derived VH-CH1 D7αRT
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protein and effective inhibition of viral retrotranscription, cells surviving after PAGO infection and GCV treatment were subsequently infected with the MMLV-LacZ retrovirus. The β-galactosidase activity in this cell population was quantitatively determined, and compared with that of the unselected population of transfectants. The results (Fig. 5 A) show that the GCV-resistant cells (VH-CH1 D7αRT PAGO selection) do indeed display a greater inhibition of β-galactosidase activity than the same cells before GCV selection (VH-CH1 D7αRT mix), confirming the correlation between expression of VH-CH1 D7αRT protein and inhibition of expression of retroviral genes. Finally, to verify that the reduction of β-galactosidase expression is due to inhibition of retroviral RT, viral DNA levels were compared in the infected cells by using a semi-quantitative PCR for the lacZ gene. Fig. 5(B) shows the calibration curves for lacZ DNA, obtained performing subsaturating PCR cycles with serially diluted lacZ DNA input. The results in Fig. 5(C) demonstrate that the levels of lacZ DNA, determined by using the calibration curves in Fig. 5(B) and normalized with respect to the α-tubulin gene, are significantly lower in VH-CH1 D7αRT transfectants than in control VH-CH1 B02αFITC-transfected cells. This inhibition is greater for
the DNA isolated from cells surviving PAGO selection (compare lanes 3–4 with lanes 5–6 in Fig. 5C), than for that isolated from unselected VH-CH1 D7αRT transfectants (compare lanes 1–2 with lanes 5–6 in Fig. 5C). The levels of viral DNA (copy number) were calculated on the basis of the DNA standard curves in Fig. 5(B), demonstrating a marked inhibition of viral retrotranscription from 750 copies per cell in the control transfectants to 20 and 170 copies in the PAGO selected and unselected transfectants, respectively. The inhibition of retrotranscription, leading to reduced proviral integration, correlates with the reduction of β-galactosidase enzymatic activity, determined before or after PAGO selection (Fig. 5A).

Altogether, these experiments strongly suggest that the VH-CH1 D7αRT antibody fragment is able to successfully inhibit the activity of retroviral RT. Furthermore, cells expressing the neutralizing phage-derived heavy chain antibody fragment are favourably selected from a background of non-expressing cells by being exposed to the selective pressure of a toxic virus, using intracellular expression of the antibody fragment as the sole selectable marker.

**Discussion**

Considerable efforts are being expended in the attempt to discover compounds that selectively inhibit HIV-1 RT, an attractive target for drug therapy of AIDS. However, prolonged chemotherapy with existing RT inhibitors, or subculturing of infected cells in vitro with increasing concentrations of drugs frequently leads to the emergence of resistant viruses, resulting from the high intrinsic mutation rate of HIV (De Clerq, 1994; Richman, 1993). For this reason, alternative therapeutic strategies based on gene therapy or on recombinant products are being actively explored (Leavit et al., 1996; Yu et al., 1994). In recent years, an experimental strategy based on the targeted intracellular expression of recombinant antibodies in mammalian cells to interfere with the function of selected molecules has been developed (Biocca & Cattaneo, 1995; Biocca et al., 1990; Cattaneo & Biocca, 1997). Following the initial feasibility studies, a number of applications of this strategy towards the creation of cellular resistance to HIV infection have been reported (Duan et al., 1994; Marasco et al., 1993; Mhashilkar et al., 1995; Shaheen et al., 1996). These studies have shown that it is indeed possible to engineer antibodies to confer protection against virus infection and replication in culture.

Most of the antibodies utilized so far for intracellular immunization have been derived from hybridomas of predefined specificity, after cloning of the corresponding genes and their engineering into more suitable formats, such as, for instance, Fab and ScFv fragments. Antibodies are increasingly being derived from phage display libraries. Among the advantages of this technology is the fact that the antibodies, most frequently ScFv fragments, are co-selected on antigen columns together with their corresponding genes, thus providing directly the recombinant version of antibodies. In order to utilize this source of recombinant antibodies for intracellular immunization schemes, we have recently exploited phage technology to isolate human synthetic antibody fragments neutralizing the enzymatic activity of HIV-1 RT (Gargano et al., 1996). The neutralization properties of the selected phage-derived antibody fragments (Gargano et al., 1996) suggested their potential use to inactivate RT by intracellular expression, in a gene therapy perspective. One of these antibody fragments (D7), which inhibits the RNA-dependent DNA polymerase activity of HIV-1 RT at nanomolar concentrations (Gargano et al., 1996), was further characterized and used for intracellular expression in mammalian cells in this work. In an attempt to evaluate the contribution of the heavy and light chains of the Fab antibody fragment D7 to RT inhibition, we discovered that the VH-CH1 heavy chain fragment alone is able to neutralize the human retroviral enzyme. The phage-derived VH-CH1 D7αRT antibody fragment also inhibits the RNA-dependent DNA polymerase activity of some HIV-1 drug-resistant RT mutants (d4T and AZT resistant). Moreover, the VH-CH1 D7αRT neutralizes the enzymatic activity of RT from murine retrovirus. On the basis of a detailed structural comparison between human and murine RTs, it has been proposed that both retroviral RTs have a conserved structure in the polymerase active site, located in the palm domains of the molecules (Georgiadis et al., 1995). Also, the structure of murine RT can be used as a model for understanding mutation in HIV-1 RT associated with nucleoside analogue resistance found in clinical isolates (Georgiadis et al., 1995). The broad neutralization properties of the phage-derived VH-CH1 D7αRT provide experimental evidence to support this structural correlation. The neutralization of both HIV-1 drug-resistant mutants and murine RTs by the phage-derived antibody fragment confirms that a conserved epitope, common to these polymerases and necessary for their enzymatic activity, is recognized by the heavy chain antibody fragment. This represents one example, of a growing number, showing binding activity by isolated heavy chains, naturally occurring camel antibodies being the prototype (Cai & Garen, 1996; Davies & Riechmann, 1995; Hamers-Casterman et al., 1993).

To verify that the synthetic antibody fragment is indeed able to interfere in vivo with RT activity, we assessed the ability of the intracellularly expressed VH-CH1 D7αRT antibody fragment to inhibit MMLV retrotranscription. By using MMLV RT and demonstrating the successful inhibition of viral retrotranscription by the intracellular expression of the antibody fragment, we have demonstrated an in vivo selection procedure, based on antibody-mediated resistance to a cytopathic retrovirus. The selective pressure exerted by the toxic virus was exploited to rescue and expand only cells expressing the anti-RT antibody fragment against a background of non-expressing cells, using resistance to the toxic virus as the sole
selection. The assay described lends itself in a natural way to select, out of a polyclonal pool of antibody specificities, intracellular antibody fragments more efficient than others in inhibiting RT. The polyclonal repertoire may be represented by a pool of mutated versions of one given antibody fragment, such as the one studied in this paper, or alternatively, by a true polyclonal mixture of antibodies derived from partial selection of a phage library on an antigen column.

This experimental strategy represents a meeting point between the technologies of phage antibody display and of intracellular antibodies and may find applications not only in virus research, but also in other research and applicative contexts.

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References


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