Rescue of a neutralizing anti-viral antibody fragment from an intracellular polyclonal repertoire expressed in mammalian cells

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Abstract The intracellular expression of recombinant antibodies in mammalian cells is an experimental strategy to inhibit in vivo the function of selected molecules. However, one limitation of this technology is represented by the unpredictable behaviour of antibodies, under conditions of intracellular expression. For this reason, it would be desirable to exploit intracellular expression of antibodies to select or rescue more efficient ones from a polyclonal mixture. In this work we have successfully explored this possibility by rescuing a neutralizing anti-viral antibody fragment from an intracellularly expressed anti-reverse transcriptase polyclonal repertoire.

Key words: Recombinant antibody; Anti-HIV antibody; Polyclonal repertoire; Mammalian cell

1. Introduction

The intracellular expression of recombinant antibodies targeted to different intracellular compartments [1,2] is an experimental strategy to interfere with the function of selected molecules. In recent years, this technology has been increasingly utilized in a variety of different systems [3,4], for research and biomedical purposes. 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, single chain antibody fragments (ScFv). However, the efficacy of an intracellularly targeted antibody fragment does not always correlate with the properties of the parental monoclonal antibody, as determined in vitro (reviewed in [5]). This is because the intrinsic overall stability and solubility of antibodies is contributed by many critical residues or combination of residues in the framework regions [6,7], and this may differently affect their folding and half-life when ectopically expressed in the reducing conditions of the cell cytoplasm. Moreover, inhibition in vivo has also been observed by antibodies that are non-neutralizing in vitro [8]. Finally, for many protein antigens an in vitro neutralization assay is not always available and an active epitope is not known a priori, making the choice of the monoclonal to be intracellularly expressed difficult. For all these reasons, it would be desirable to exploit intracellular expression of antibodies to select or rescue more efficient ones from a polyclonal mixture. While the monoclonal antibody technology does not easily allow to handle recombinant versions of polyclonals, new antibody technologies seem more suitable in this respect. Antibodies are increasingly being derived from phage display libraries [9]. Among the advantages of this technology is the fact that the antibodies, most frequently ScFv fragments, are coselected on antigen columns together with their corresponding genes. Therefore a polyclonal population of antigen binding phage also provides the genes for the corresponding affinity purified polyclonal antibodies.

We have recently provided the first example that phage derived antibody fragments can be successfully used for intracellular immunization [10]. We showed that an antibody fragment derived from a large phage display library, directed against the reverse transcriptase of human immunodeficiency virus (HIV-1 RT), can inhibit viral retrotranscription, upon intracellular expression as a cytosolic protein. In this paper we explore the possibility of rescuing an anti-viral neutralizing antibody, from an intracellularly expressed polyclonal repertoire, by imposing a selective pressure of a cytotoxic retrovirus. This would allow to extend the intracellular expression of single antibody specificities, to that of more diverse repertoires.

2. Materials and methods

2.1. Selection and screening of anti-HIV-1 RT phage antibody fragments

Recombinant HIV-1 RT [11] was used to select single chain Fv fragments (ScFv) from phage display libraries of human Fab [12] and ScFv fragments [13]. The selection from the Fab library was previously described [11]. The selection from the ScFv library was performed as described [13], through three cycles of phage growth and selection with HIV-1 RT coated on immunotubes (Nunc), at a concentration of 10 μg/ml in phosphate buffered saline (PBS). Phage-ELISA on solid phase HIV-1 RT (10 μg/ml in PBS) was performed as described [12], using either a sheep anti-fd antisera (for the Fab library) or an anti-M13 antisera (for the ScFv library), followed by a peroxidase conjugated secondary antibody. The RT RNA-dependent DNA-polymerase activity was measured by mixing 250 ng/ml recombinant HIV-1 RT and 5×10^{11} phage particles/ml in reaction buffer (PBS, 2.5 mM $MgCl_2$, 1.5 mM [α-^{32}P]dGTP, 10 μg/ml poly-rC/oligo-dG), in a total volume of 20 μl. After preincubation of RT and phages for 20 min on ice, the reaction was started at 37°C and terminated after 30 min by adding 1 ml of ice cold PBS. The mix was then filtered on DE81 chromatographic paper, followed by washing of the filters three times with 5% Na_2HPO_4 and once with water. Radioactivity bound to the filters was finally counted.

2.2. Plasmids, cell cultures and viral challenge

For the expression of antibody fragments in mammalian cells, plasmids pScFvexp-D7αRT and pScFvexp-polyαRT were constructed by subcloning *Nco*I-*Not*I fragments from pUC119-FabD7 [11] and pCANTAB-6-polyαRT (third round of selection) [13] respectively, into *Nco*I-*Not*I cut pScFvexp. These vectors direct the expression of cytosolic antibody fragments under the transcriptional control of the EF-BOS (elongation factor-1a) promoter [14]. Murine fibroblast Balb/c-3T3 cells were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS). Transfection of Balb/c-3T3 cells with pScFvexp-D7αRT and pScFvexp-polyαRT vectors was performed

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by electroporation: cells were trypsin harvested, washed twice with PBS and resuspended in 0.3 ml of DMEM medium (1.3×10^7 cells/ml) to which 10 μg of 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 resuspended in 10 ml of DMEM/10% FCS and incubated for 24 h at 37°C. Transfected cells were collected after two weeks of neomycin selection (G418 1 mg/ml) in DMEM/10% FCS.

The B16-PAGO ecotropic retrovirus packaging cell line, kindly provided by R. Vile, was used as a source of the Moloney-based MMLV-PAGO replication defective viruses [15]. This retrovirus encodes for the expression of the herpes simplex virus thymidine kinase (HSV-tk) gene, which confers to cell sensitivity to the toxic action of Gancyclovir (GCV). The packaging cell line was grown in DMEM/10% FCS supplemented with neomycin (2.5 mg/ml). For the infection of fibroblast Balb/c-3T3 cells with PAGO virus, a total of 5×10^5 cells were incubated with 10 ml of supernatant of subconfluent B16-PAGO packaging cells (12 h of production of MMLV-PAGO virus; titer 10^5 CFU/ml) for 5 h with Polybrene (4 μg/ml). After the infection, the cells were maintained for 24 h in growth medium and then incubated in the presence of GCV (10 μg/ml) for 2 weeks, changing the medium every 3 days. The antibody fragment D7αRT was detected in cell transfectants by Western analysis with the anti-myc-tag monoclonal antibody 9E10, as described [10].

2.3. *Rescue of the neutralizing anti-RT antibody fragment DNA under viral selective pressure*

Balb/c-3T3 cells, transfected with a mixture of pScFvexp-D7αRT and pScFvexp-polyαRT DNA (ratio from 10^{-2} to 10^{-3}), were infected with PAGO virus as above. After two weeks of GCV selection, surviving cells were washed with PBS, and the level of the D7αRT and ScFv-polyαRT antibody fragment DNA in the cell population was monitored by using a semi-quantitative PCR analysis. Total cellular DNA was prepared by a quick lysis method. Briefly, 10^5 cells were solubilized in 100 μl of lysis buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% NP40, 0.5% Tween20, 50 μg/ml proteinase-K) and incubated for 1 h at 56°C. The samples were then boiled for 10 min to inactivate the proteinase-K. The D7αRT DNA was amplified by using a 5′-primer in the CH1 region (human IgG1) and a 3′-primer in the ScFv-express vector myc-tag region. The polyαRT DNA was detected with a 5′-primer in the ScFv linker region and the same 3′-primer as above. To normalize for cellular DNA, mouse α2-tubulin DNA was amplified with 10-fold diluted samples. The PCR products were separated on 1.5% agarose gels, transferred onto Hybond-N^+ membranes (Amersham), hybridized with 5′-end-labeled ^{32}P-oligonucleotide probes and revealed by autoradiography. A subsaturating number of PCR cycles, as determined from preliminary experiments, was performed (22 cycles were routinely performed).

3. Results

To rescue an anti-viral neutralizing antibody from an intracellularly expressed polyclonal repertoire, the reverse transcriptase (RT) was utilized as the target antigen and resistance to the killing action of a cytotoxic retrovirus as the selectable phenotype. We have recently described the isolation from a synthetic library of Fab fragments [12] of one antibody fragment that neutralizes the RNA-dependent DNA-polymerase activity of HIV-1 RT, as well as that of RT from other retrovirus [11]. A further characterization of the phage derived Fab fragment D7αRT showed that the heavy chain alone (D7αRT) of this Fab fragment retains the ability to neutralize the activity of RTs of diverse origin [10]. The intracellular expression of the D7αRT antibody fragment in the cytoplasm of mammalian cells leads to an efficient inhibition of viral retrotranscription by murine retroviruses [10].

As a prelude to intracellular library experiments, we performed a model cell selection. To this aim, cells expressing the D7αRT antibody fragment were diluted with an excess of negative cells and the cell population was challenged with the PAGO retrovirus, a Moloney-based replication defective retrovirus carrying the herpes virus thymidine kinase (HSV-tk) as reporter gene [15]. The expression of HSV-tk converts the drug gancyclovir (GCV) into a toxic compound, thus leading to GCV-dependent cell death. After infection, the cell population was cultured with GCV and the levels of D7αRT DNA and protein were monitored at different times of incubation with GCV. The results in Fig. 1A (Southern analysis of PCR products) clearly show that the DNA encoding for D7αRT (top) is progressively enriched, as a function of incubation time with GCV, with respect to a housekeeping

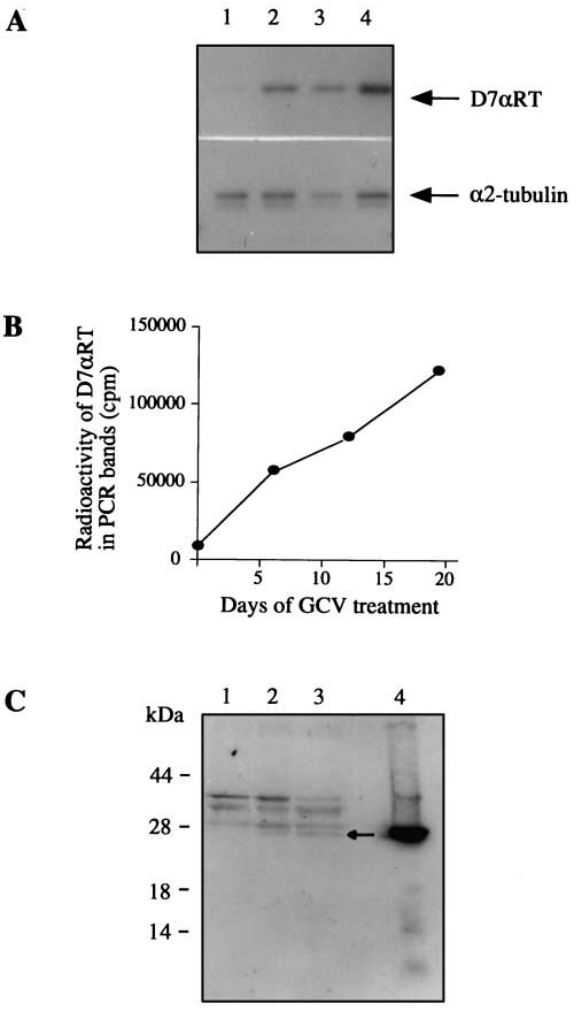

Fig. 1. Rescue of D7αRT expressing cells under gancyclovir selective pressure. D7αRT expressing Balb/c3T3 cells were mixed at a ratio of 10^{-2} with untransfected cells and infected with PAGO viruses. At different times after PAGO infection and GCV exposure, cells were collected and the levels of D7αRT protein and DNA assayed. A: Southern analysis of PCR products. Total cellular DNA (10^4 cell equivalents/lane) was amplified by PCR with primers complementary to the D7αRT and α2-tubulin genes and hybridized as described in Section 2. Cell samples derived before gancyclovir treatment (lane 1), after 6 (lane 2), 12 (lane 3) and 20 days (lane 4) of GCV exposure were analyzed. B: Amount of radioactivity (Phosphorimager) in the hybridized D7αRT DNA bands, normalized to α2-tubulin. C: Western analysis of D7αRT protein during GCV selection. 2.5×10^5 cells were collected before gancyclovir exposure (lane 1), after 6 (lane 2), or 12 days of GCV treatment (lane 3). The D7αRT protein was visualized with Mab 9E10. D7αRT control protein from baculovirus infected insect cells (lane 4).

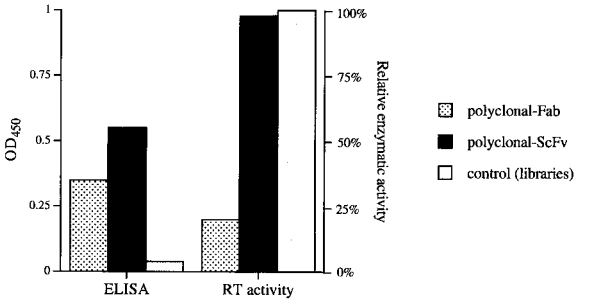

Fig. 2. Selection of anti HIV-1 RT phage antibodies. Polyclonal anti-RT phage antibodies, selected from the Fab library [12] (shaded bars) or from the ScFv library [13] (filled bars), were assayed by ELISA for binding to HIV-1 RT in solid phase. The same polyclonal phage antibodies were assayed for their ability to inhibit the RNA-dependent DNA-polymerase activity of HIV-1 RT. An equal number of phages derived from the unselected libraries was analyzed as controls (gray bars).

gene (α2-tubulin, bottom). Normalization to the α2-tubulin gene yields the values shown in Fig. 1B. Thus, cells harbouring the D7αRT gene are progressively enriched in the population. This enrichment of D7αRT DNA was further confirmed at the level of the corresponding protein, as shown by Western analysis of cell extracts taken at different times of GCV selection (Fig. 1C). These results demonstrate that cells expressing the D7αRT antibody fragment, can be efficiently rescued and expanded after two weeks of selection with GCV, using intracellular expression of the antibody fragment as the sole selectable marker.

In order to explore the possibility of rescuing an anti-viral neutralizing antibody, from an intracellularly expressed polyclonal repertoire, we performed a new antibody selection, using recombinant HIV-1 RT [11] as a solid phase selector of a naive repertoire of human ScFv fragments [13]. This led, after three rounds of selection, to the isolation of a polyclonal population of phage-displayed ScFv fragments which reacted strongly with HIV-1 RT in ELISA. Single clones were grown, showing that approximately 10% of the individual phages were RT positive in ELISA. Fingerprinting analysis of the V-genes of ELISA-positive clones, amplified by PCR, showed a diversity in the order of 15 different ScFvs (data not shown). The polyclonal anti-RT phage population did not show any significant inhibition of RT activity in vitro (Fig. 2), under conditions in which the polyclonal phage population containing phage D7αRT (seventh cycle of selection, [11]) exerted a strong inhibition.

We then sought to study the in vivo properties of the anti-RT antibody fragments. To this aim, the bulk DNA encoding for the third round polyclonal ScFv-polyαRT fragments was subcloned into the ScFv-express vector [14], the same expression vector in which the neutralizing D7αRT antibody fragment was previously cloned for expression in the cytoplasm of mammalian cells [10]. In order to perform a model selection from an 'intracellular library', the D7αRT DNA was mixed with increasing amounts of the polyclonal ScFv-polyαRT DNA. These DNA mixtures were transfected into Balb/c-3T3 cells and, after two weeks in selective medium (neomycin), cells were challenged with the PAGO retrovirus and cultured with GCV. As shown in Fig. 3A, the number of surviving clones after GCV selection correlates closely with the input D7αRT DNA in the transfection mixture, suggesting that cells expressing this neutralizing antibody fragment are being rescued, at the expense of cells expressing other antibody fragments. In order to verify whether the GCV resistance correlates with the presence of the D7αRT in the surviving cells, genomic DNA was extracted from the surviving cell population and the presence of genes encoding for polyαRT and for the D7αRT fragments was assessed by semi-quantitative PCR analysis with specific oligonucleotides that distinguish between the two antibody formats. The results are shown in Fig. 3B, demonstrating that while the gene encoding the neutralizing antibody fragment is enriched as the GCV selection proceeds, DNA encoding for the non-neutralizing ScFvs is being lost. It is noteworthy that the D7αRT DNA is enriched even when it was only present at a 10^{-3} dilution in the transfection mixture.

These results confirm that only the cells expressing the neutralizing antibody fragment can be efficiently rescued and expanded after two weeks of selection with GCV, using intracellular expression of the antibody fragment as the sole selection. Thus, this selection procedure can be used to rescue, in vivo, a neutralizing antibody fragment from a polyclonal background of non-neutralizing ones.

4. Discussion

These findings demonstrate that the intracellular expression in mammalian cells of a polyclonal repertoire of antibody fragments can be used to select antibodies on the basis of a given function. In fact, these experiments show that the ability of a neutralizing anti-reverse transcriptase antibody fragment

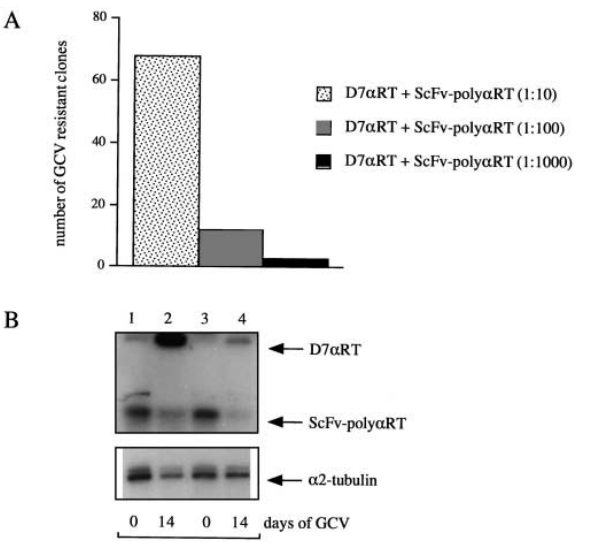

Fig. 3. Rescue of the neutralizing anti-viral antibody fragment from an intracellular library. A: Survival of Balb/c-3T3 cells transfected with a mixture of D7αRT and ScFv-polyαRT DNA at different ratios (D7:ScFv = 1:10, shaded bar; 1:100, empty bar; 1:1000, filled bar). Transfected cells were infected with PAGO virus, incubated for two weeks with GCV and the number of GCV resistant clones was determined. The values are the mean of two independent experiments. B: Southern analysis of PCR products. Balb/c-3T3 cells, transfected with mixtures of D7αRT and ScFv-polyαRT DNA (lanes 1–2, D7:ScFv = 1:100; lanes 3–4, D7:ScFv = 1:1000), were challenged with PAGO and incubated with GCV. Before infection (lanes 1 and 3) and after 14 days of GCV treatment (lanes 2 and 4), cellular DNA was amplified (10^4 cell equivalents/lane) with primers specific for D7αRT, SvFv-polyαRT and cellular α2-tubulin.

to confer cell resistance to a toxic retrovirus can be used in a selection scheme for its rescue against a background of non-neutralizing ones, directed against the same protein. Intracellular antibody expression has always been performed, so far, with genes derived from monoclonal antibodies [2,3] or with phage derived antibody fragments [10] of a well defined specificity. The use of phage derived antibody fragments has allowed us to provide a 'proof of principle' for the concept of intracellular libraries, whereby an affinity purified polyclonal population of antibody fragments is intracellularly expressed. The design of a suitable selection scheme should allow the rescue of antibody specificities from the polyclonal repertoire. This strategy can be illustrated for the case of resistance to a cytotoxic virus, since this closely reflects the experimental design utilized for this work. A large phage display library of antibody fragments is challenged with an antigen (Ag) involved in the viral life cycle and an affinity purified population of phages, enriched in anti-Ag specificities, and of greatly reduced diversity, is selected after few cycles. Bypassing the in vitro characterization of individual antibodies in this polyclonal population, this small enriched repertoire is formatted for intracellular expression using appropriate vectors [14], and is transfected as a pooled DNA in mammalian cells. Individual cells will express single specificities, or even a small subset of antibody specificities. By exposing the cell population to the selective pressure of a cytotoxic retrovirus, those antibody specificities interfering with the retroviral life cycle will confer a selective advantage to the cells harbouring them. These cells will therefore outgrow the culture. Finally, the neutralizing antibody fragments can be rescued from these cells and characterized. This scheme is rather broad, and need not be restricted to the phenotypic resistance to a cytotoxic virus. In fact, it should work, in principle, for all those cases were intracellular antibodies could provide a growth advantage to cells expressing them. For instance, the selection of antibodies against apoptotic proteins, should be possible by this procedure. This approach is not limited to cytoplasmic expression, but can be applied to a number of different intracellular compartments, provided a suitable selection scheme is available (NG and AC, unpublished data). It is clear however, as we are dealing with mammalian cells, that the size of the repertoire cannot be too large. Hence, the need of a preselection step, such as, for instance, an affinity purification on antigen column. We envisage that a repertoire size in the order of 10^3–10^4 is compatible with selection schemes in mammalian cells, not limiting the sensitivity of the assay to screen all possible candidates. For this reason, the experiments were performed with a dilution ratio of 10^{-3}, and no attempt was made to improve on this, although it would have been possible, for instance by increasing the number of cells or the selection time. The polyclonal repertoire can also be restricted in alternative ways, such as for instance a small mutated library of a lead antibody fragment. This may allow selection for higher affinity or for improved intracellular performance to be achieved in vivo. Also, a number of procedures to enrich in specific binders of interest from a large library of antibodies could be used: for instance (i) subtraction methods for the enrichment of phage antibodies against proteins constituting a difference between two population of cells [16], or (ii) methods for panning of phage libraries on living cells [17]. The approach outlined in this paper can only be implemented by exploiting the phage technology, since only in this case it is possible to easily handle the recombinant version of a polyclonal antiserum. The design of a suitable selection scheme should allow to select antibodies against known or unknown epitopes. In conclusion, with antibodies derived from large display libraries, the intracellular targeting of polyclonal repertoires in mammalian cells is now feasible.

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