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Human Recombinant Antibody Fragments Neutralizing Human Immunodeficiency Virus Type 1 Reverse Transcriptase Provide an Experimental Basis for the Structural Classification of the DNA Polymerase Family

NICOLA GARGANO,[1,2] SILVIA BIOCCA,[3] ANDREW BRADBURY,[1]
AND ANTONINO CATTANEO[1,2]*

International School for Advanced Studies, Trieste,[1] and Institute of Neurobiology, Consiglio Nazionale delle Ricerche, Rome,[3] Italy, and Laboratory of Molecular Biology, Medical Research Council, Cambridge, United Kingdom[2]

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We describe in this paper the binding and biochemical properties of two human antibody fragments directed against the human immunodeficiency virus type 1 reverse transcriptase (RT). These fragments were isolated from a synthetic combinatorial library of human Fab antibody fragments displayed on the surface of filamentous phage. The antibody fragments were selected by using recombinant heterodimeric human immunodeficiency virus type 1 RT purified from insect cells as a solid-phase selector. This procedure led to the isolation of two antibody fragments that completely neutralize the RNA-dependent DNA polymerase activity of RT at nanomolar concentrations. Both antibody fragments bind only to the enzymatically active form of the RT. The inhibitory activity of the anti-RT antibody fragments is competitive with respect to the template primer. The antibody fragments also neutralize the activities of RTs from avian and murine retroviruses and of DNA polymerases of prokaryotic origin as well as human DNA polymerase alpha. Thus, the antibody fragments selected and characterized in this study appear to recognize a structural fold that is common to the different DNA polymerases and necessary for their activity. The results provide an immunological experimental basis for a purely structural and evolutionary classification of the polymerase family.

Considerable effort is being expended in the attempt to discover compounds that inhibit human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), which is responsible for the conversion of the (+) single-stranded RNA viral genome into double-stranded DNA, which subsequently becomes integrated into host cell chromosomes. RT is an attractive target for drug therapy, not only because it is essential for HIV replication but also because it is not required for normal host cell metabolism. Inhibitors that target RT fall within two broad categories: nucleoside analogs and nonnucleoside derivatives (for reviews, see references 20 and 34). However, prolonged chemotherapy, or passage of infected cells in vitro with increasing concentrations of drugs, results in the emergence of resistant viruses. This resistance is due to specific changes within the *pol* gene (6, 30), resulting from the high intrinsic mutation rate of HIV, which leads to viral genetic heterogeneity. The recent resolution of the RT structure, in isolation or complexed with a number of these inhibitors (17, 19, 26, 27), has allowed these escape mutations to be put into a structural context (34) and has allowed insight into the mechanisms of enzyme action (25) and of resistance to these drugs (9).

For this reason, alternative therapeutic strategies based on gene therapy or on recombinant products are being actively explored (39). In recent years, an experimental strategy based on the targeted expression of recombinant antibodies to intracellular compartments of mammalian cells has been developed (3, 4). Following the initial feasibility studies, applications of this strategy towards the creation of cellular resistance to the HIV infection have been recently reported (8, 21, 22, 24). These studies showed that in vitro it is indeed possible to engineer antibodies to confer protection against viral infection and replication.

In order to turn a promising experimental technology into a therapeutically applicable one, several criteria need to be met. In particular, high-affinity, broadly neutralizing antibodies of human origin need to be available in recombinant form. With this in mind, in order to isolate an antibody suitable for its intracellular expression, we exploited phage technology (37) and utilized a large combinatorial library of human Fab antibody fragments expressed on the surface of filamentous phage (14). In this paper we describe the isolation and characterization of two antibody fragments that neutralize the RNA-dependent DNA polymerase activity of HIV-1 RT as well as the activities of other polymerase enzymes of diverse origins.

MATERIALS AND METHODS

Plasmid and recombinant baculovirus construction. *Autographa californica* multiple nuclear polyhedrosis virus strain E2 and the baculovirus expression vector pVL1392 were from Pharmingen Laboratories. Plasmids pGEM-RT51 and pGEM-RT66, kindly provided by R. Sobol, were used as a source of DNA encoding the p51 and p66 subunits of HIV-1 RT. Recombinant baculoviruses harboring HIV-1 RT subunit-encoding sequences (BV-RT51 and BV-RT66, respectively) were constructed by cotransfection of plasmids pVL1392-RT51 and pVL1392-RT66, respectively, with *A. californica* multiple nuclear polyhedrosis virus DNA into *Spodoptera frugiperda* (Sf9) cells. Plasmids pVL1392-RT51 and pVL1392-RT66 were constructed by subcloning *Hin*dIII-*Eco*RI fragments from pGEM-RT51 and pGEM-RT66, respectively, blunted at the *Hin*dIII sites, into *Sma*I-*Eco*RI-cut pVL1392. Recombinant baculoviruses able to induce expression of the HIV-1 RT subunits were isolated by plaque purification from Sf9 cells infected with the supernatant of the primary cotransfection, as previously described (33). Sf9 cells were grown in TNM-FH medium (Sigma) with 10% heat-inactivated fetal calf serum at 27°C. Anti HIV-1 RT monoclonal antibody 1E8 (38) was a kind gift of R. Sobol.

Purification of HIV-1 RT heterodimers expressed in insect cells. Sf9 cells (10^9) were coinfected with BV-RT51 and BV-RT66 baculoviruses (multiplicity of

* Corresponding author. Mailing address: MRC Laboratory of Molecular Biology, Hills Rd., Cambridge CB2 2QH, United Kingdom.

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infection, 5 PFU of each virus per cell) and harvested 72 h later. After being washed with phosphate-buffered saline (PBS), cell pellets were resuspended in 30 ml of ice-cold extraction buffer (EB) (20 mM Tris [pH 8.0], 20 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, protease inhibitor cocktail), homogenized with a Dounce homogenizer (30 strokes, 4°C), and centrifuged for 30 min at 10,000 × *g*. The supernatant was kept aside, and the cell debris were reextracted with 20 ml of EB as described above. The pooled supernatants were chromatographed at 4°C through DEAE-cellulose (Pharmacia) (20-ml packed volume) and heparin-agarose (Pharmacia) (2.5-ml packed volume) columns tandemly connected. Both columns were equilibrated in EB, and the flow was 20 ml/h. After being washed with 50 ml of EB, the columns were disconnected, and the heparin-agarose column was washed with 25 ml of EB plus 50 mM NaCl. RT molecules bound to the heparin column were eluted with 8 ml of EB plus 400 mM NaCl. The eluted pool was made to 80 ml with TEDN buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol, 40 mM NaCl) and loaded on a single-stranded-DNA–agarose column (Sigma) (2-ml packed volume), equilibrated in TEDN (15-ml/h flow). The single-stranded DNA column was washed with 10 volumes of TEDN buffer and eluted with an NaCl step gradient of 100, 200, 300, 400, and 500 mM in TEDN (2 ml per NaCl step), and 0.5-ml fractions were collected and frozen, after analysis, at −20°C in 10% glycerol. The specific activity of the high-salt-eluted fraction (300 to 400 mM NaCl) was equal to 320 ± 10 nmol of dGTP per min per mg of recombinant protein. This value correlates well with that obtained for RT heterodimers purified from *Escherichia coli* cells (250 to 300 nmol of dGTP per min per mg) (38). The K_m of the purified enzyme, as determined by an Eadie-Hofstee plot with the concentration of the poly(rC)-oligo(dG) substrate varied, was found to be 6.0 ± 0.2 μg/ml of template, which was somewhat higher than that previously reported for the enzyme purified from *E. coli* (2.0 ± 0.3 μg/ml) (15).

With this procedure the recovery of HIV-1 RT heterodimer was 2 mg from 10^9 Sf9-coinfected cells.

Selection and screening of anti-HIV-1 RT phage antibodies from the combinatorial phage display library. The phage display library of human Fab fragments (14) was kindly provided by A. Griffiths and G. Winter. HIV-1 RT protein purified from Sf9 cells was applied to immunotubes at a concentration of 10 μg/ml in PBS. The selection procedure was performed essentially as described previously (14), with minor variations described below.

The number of input phage for the first round of selection was 10^{13}, obtained by growing in *E. coli* TG1 cells 10^{10} phage from the original Griffiths library (half of which were from the recombined lambda and half from the recombined kappa light-chain-derived library). The number of input phage for the successive rounds of selection was 10^{11}. Selection cycles of panning, washing, elution, and growth of eluted phage were performed essentially as described previously, through seven cycles of growth and selection, except that after the second and the fourth cycles, phage recovered after growth on 2× TY-TET agarose plates were subjected to a second growth phase on 2× TY-TET agarose plates instead of to the usual growth in liquid medium.

Phage enzyme-linked immunosorbent assay (ELISA) on solid-phase-coated HIV-1 RT was performed as previously described (14), using a sheep anti-fd antiserum (kindly provided by P. Jones) followed by a peroxidase-conjugated secondary antibody (Sigma).

For soluble expression of antibody fragments, DNA from individual phage colonies was amplified as described previously (14) and cloned into pUC119 His6mycXba, kindly provided by A. Griffiths. After transformation of *E. coli* TG1 cells, expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) as described previously (23). Antibody fragments were purified via their histidine tag on Ni-nitrilotriacetic acid resin (Qiagen) from the periplasmic fraction and the osmotic shock fraction, as described by the manufacturer (Qiagen manual). 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; cutoff, 10 kDa) and suspended in PBS by repeated washing of the filters. The antibody proteins were kept frozen in aliquots at 100 μg/ml. Purified antibody fragments were assayed by ELISA for their ability to bind to solid-phase-bound RT (applied at 10 μg/ml in PBS). The products of the binding reaction were revealed with a mixture of anti-human kappa, anti-human lambda, and anti-human gamma chain rabbit antisera (kindly provided by R. Marzari) followed by peroxidase-conjugated anti-rabbit antiserum (Cappell).

Enzymatic assays for RNA-dependent DNA polymerase activity of HIV-1 RT. RNA-dependent DNA polymerase activity was measured in an assay mixture consisting of 500 ng of recombinant HIV-1 RT per ml in RB buffer [PBS, 2.5 mM $MgCl_2$, 1.5 μM [α-^{32}P]dGTP, 10 μg of poly(rC)-oligo(dG)], 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, the mixture was filtered on DE81 chromatographic paper, and the filters were washed three times with 5% Na_2HPO_4 and once with water. The radioactivity bound to the filters was finally counted.

When the activity of anti-RT antibodies or antibody fragments was monitored, these were preincubated with the RT protein in RB buffer for 10 min on ice, and then [α-^{32}P]dGTP and poly(rC)-oligo(dG) were added and the incubation at 37°C was started. In the case of phage antibodies, 5 × 10^{11} phage particles per ml were incubated with 250 ng of recombinant HIV-1 RT per ml for 10 min on ice, in a volume of 20 μl of RB, before the reaction was started as described above.

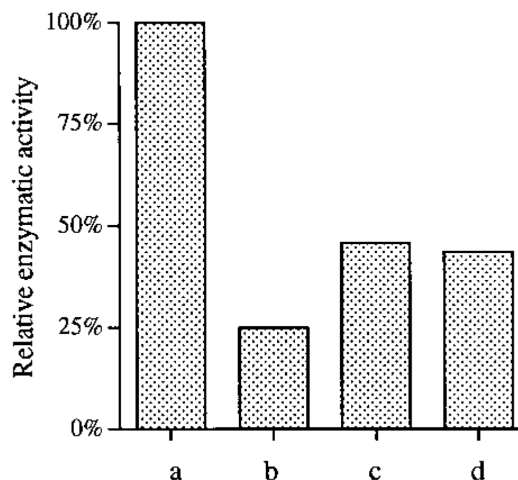

FIG. 1. Selection of HIV-1 RT-neutralizing antibody fragments from a phage display library. Inhibition of RNA-dependent DNA polymerase activity of recombinant HIV-1 RT by selected polyclonal and monoclonal phage is shown. p51/66 RT was incubated in the presence of phage antibodies derived from the unselected library (bar a), polyclonal phages after seven rounds of RT selection (bar b), and two selected phages (phage C7 [bar c] and phage D7 [bar d]). RT RNA-dependent DNA polymerase activity was assayed under standard conditions as described in Materials and Methods.

The assay for other retroviral RT enzymes was performed under identical conditions, using commercial enzymes. Moloney murine leukemia virus RT was purchased from Promega as an RNase H-deficient enzyme. Avian myeloblastosis virus RT was from Promega. The DNA polymerase activities of the Klenow fragment of *E. coli* DNA polymerase I (Boehringer) and of T7 DNA polymerase (Promega) were assayed by standard random primer extension (Random primed kit; Boehringer). The DNA polymerase activity of terminal deoxynucleotidyl transferase (Promega) was measured under standard conditions (31). Human DNA polymerase alpha (pol α) and rat DNA pol β were kindly provided by Dave Stammers and Zdenek Hostomsky, respectively, and were assayed by using a poly(dA)-oligo(dT) template primer.

RESULTS

Selection of anti-HIV-1 RT human antibody fragments from a combinatorial phage display library. The HIV-1 RT protein purified from Sf9 cells was used for the selection of human antibody fragments from a combinatorial library of synthetic repertoires of human heavy and light chains displayed on filamentous phage (14). After the HIV-1 RT was applied to the solid phase, the selection was performed by successive rounds of panning, elution, and phage growth, and the presence of phage particles with a binding specificity for HIV-1 RT was directly monitored after each cycle by phage ELISA. Enrichment of RT-positive phage particles was found after six selection rounds, as measured on the "polyclonal" phage population. At the end of the selection process (seven cycles), single clones of phage antibody particles were isolated, and more than 50% of them were shown to bind RT by phage ELISA (not shown).

Both the polyclonal and individual ELISA-positive "monoclonal" phages were assayed for the ability to interfere with the RT enzymatic activity. The results in Fig. 1 show that both the polyclonal and monoclonal phage antibodies markedly inhibit the RNA-dependent DNA polymerase activity of recombinant RT (up to 50% under these conditions), with respect to an equal number of phages from the initial unselected library. It is noteworthy that this enzymatic assay was performed with both phage and enzyme at nanomolar concentrations, pointing to a high-affinity interaction between the phage antibodies and the RT.

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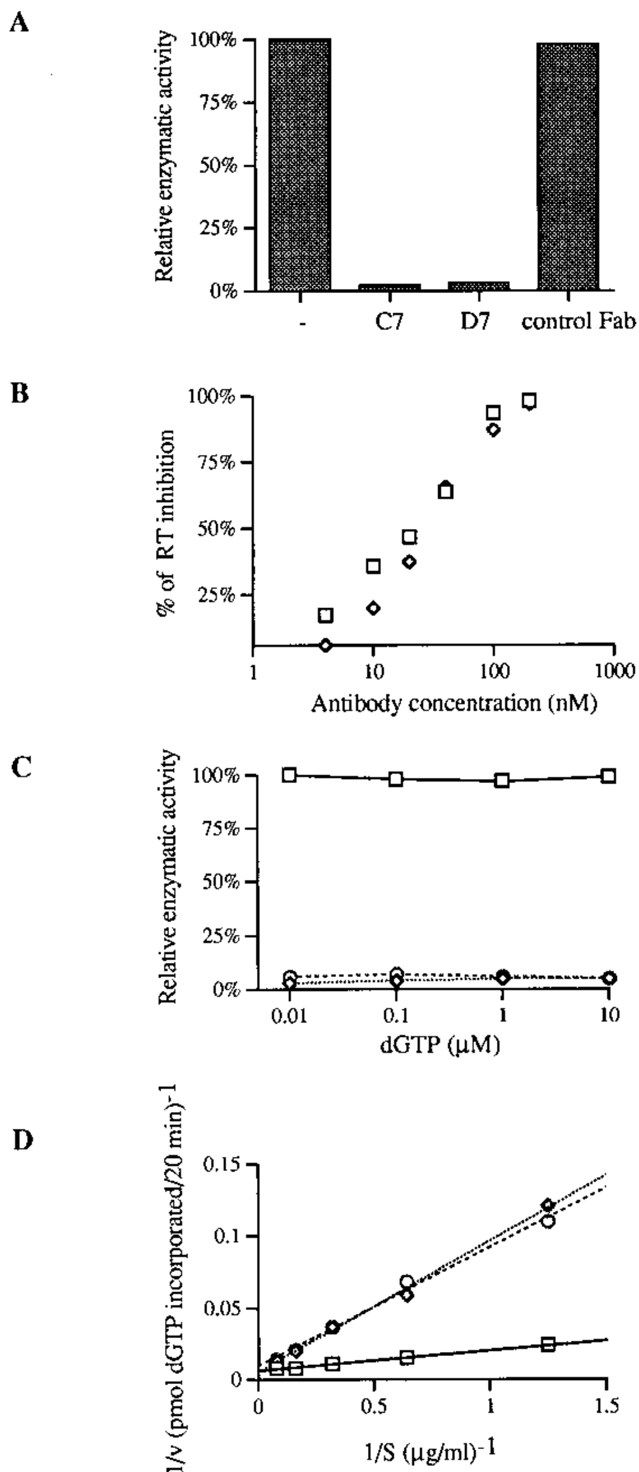

FIG. 2. Biochemical characterization of anti HIV-1 RT antibody fragments C7 and D7 expressed in *E. coli* as soluble proteins. HIV-1 RT RNA-dependent DNA polymerase activity was assayed under standard conditions, as described in Materials and Methods, in the presence of control Fabs and the selected anti-RT antibody fragments C7 and D7. In all experiments the concentration of RT was 4 nM. The concentration of the antibody fragments was 100 nM for panels A, C, and D; that of the poly(rC)-oligo(dG) template primer was 10 μg/ml for panels A, B, and C; and that of dGTP was 10 μM for panels A, B, and D. (A) Inhibition of the RNA-dependent DNA polymerase activity of HIV-1 RT by selected antibody fragments C7 and D7. (B) Effect of antibody fragment concentrations on the inhibition of the activity of HIV-1 RT by C7 (□) and D7 (◇). (C) Inhibition of the enzymatic activity of HIV-1 RT by selected antibody fragments C7 (◇) and D7 (○) at different dGTP concentrations. □, control Fab. (D) Inhibition of the RNA-dependent DNA polymerase activity of HIV-1 RT by antibody fragments C7 (◇) and D7 (○) at different template primer concentrations. The results are plotted as a double-reciprocal plot and show that the inhibition of RT activity by C7 and D7 is competitive with respect to the poly(rC)-oligo(dG) template primer. □, control Fab.

Expression and biochemical characterization of the neutralizing anti-HIV-1 RT antibody fragments. Two phage antibodies that were most active in the inhibition of RT enzymatic activity (phages C7 and D7) were chosen for further genetic and biochemical characterization. Sequencing of the DNA coding for the antibody fragments showed that both belong to the VH3 family of human heavy-chain-variable regions (36) while having different complementarity-determining region 3 (CDR3) sequences (data not shown). Antibody fragments were expressed as soluble proteins in *E. coli* and purified from the periplasmic space by using the histidine tag present at the C terminus of the heavy chain. The influence of the soluble antibody fragments on the enzymatic activity of HIV-1 RT was tested, and the results (Fig. 2A) confirmed that, like the phage particles C7 and D7 from which they were derived, the corresponding soluble antibody fragments display a complete inhibition of the RT enzymatic activity. A control Fab fragment, expressed and purified under the same conditions, induces no inhibition of RT activity. In this experiment the concentration of the RT enzyme was 4 nM, and that of the antibody fragments was 100 nM. A wider range of antibody concentrations, at constant concentrations of RT (4 nM) and of the poly(rC)-oligo(dG) substrate (10 μg/ml), was explored in the experiment reported in Fig. 2B. The two antibody fragments display similar inhibition curves, with a 50% neutralization under these conditions at an antibody concentration of around 20 to 30 nM.

We then determined the effect of varying the concentrations of dGTP and of the poly(rC)-oligo(dG) substrate on the inhibition of RT enzymatic activity by the two antibody fragments. The results in Fig. 2C show that the inhibition of RT activity is not reversed by increasing the dGTP concentration over a range of 3 orders of magnitude, therefore suggesting a possible noncompetitive inhibition. In contrast, the inhibition of RT activity by both antibody fragments is highly sensitive to the concentration of the poly(rC)-oligo(dG) substrate, as demonstrated in Fig. 2D, where the results are reported in the form of a double-reciprocal plot. This experiment provides evidence for a mechanism of competitive inhibition with respect to the RNA substrate, which is similar for the two antibody fragments. Both antibody fragments increase the K_m for the template primer by a factor of 6. The same value for the K_m increase caused by the two antibody fragments is obtained by plotting the data as an Eadie-Hofstee plot. This would correspond to a K_i in the range of 5 to 10 nM. It is therefore likely that the two antibody fragments bind to a common epitope, thereby occluding, or displacing, the binding of the substrate. In conclusion, on the basis of the results in Fig. 2, it is reasonable to estimate that the affinity of the selected antibody fragments for RT is comparable to or higher than that of the template primer for RT, which is 50 nM (2).

Experiments with ^{125}I-labelled RT showed binding of the antibody fragments to ^{125}I-RT and confirmed that the two antibody fragments compete with each other for a common epitope on the enzyme (data not shown). In order to gain insight into the epitope recognized by the two selected antibody fragments C7 and D7, these fragments were challenged with a series of overlapping 10-amino-acid peptides encom-

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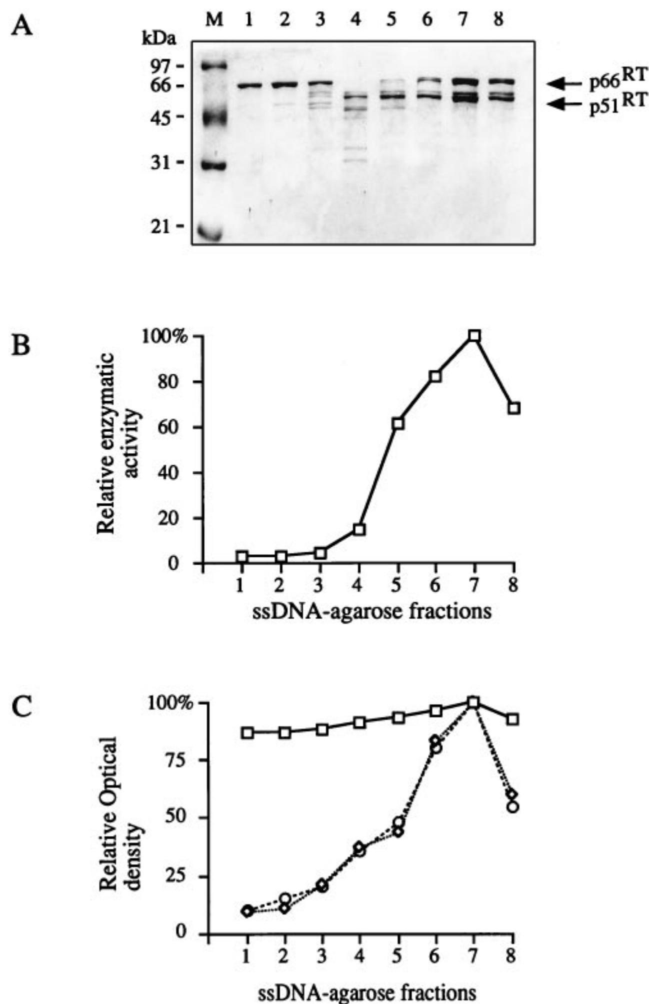

FIG. 3. Antibody fragments C7 and D7 bind selectively to the active form of RT. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the fractions eluted from a single-stranded DNA (ssDNA)-agarose column. Lane M, molecular mass markers. (B) Enzymatic RNA-dependent DNA polymerase activity of the corresponding fractions. (C) Fractions were used to coat ELISA plates (coating concentration, 10 μg/ml in PBS), and solid-phase-coupled RT subunits were challenged with 1 μg of purified mouse monoclonal antibody 1E8 per ml (□) or with 1 μg of purified antibody fragment C7 (○) or D7 (◇) per ml followed by rabbit anti-human gamma chain antibodies and by peroxidase-conjugated goat anti-rabbit antibodies.

passing the whole of the RT molecule, coupled to a solid phase. No positively reacting peptide was found (data not shown) under conditions in which native full-length RT reacted strongly, thus indicating that the two antibody fragments do not recognize linear epitopes.

Both antibody fragments bind only to the active form of the RT enzyme and fail to bind to the inactive RT subunits. This conclusion was reached by exploiting RT protein fractions (Fig. 3A) that are enzymatically active (fractions 5 to 8 in Fig. 3B) or inactive (fractions 1 to 4 in Fig. 3B). Figure 3C shows that only the enzymatically active fractions, fractions 5 to 8, are recognized in ELISA by the antibody fragments D7 and C7, while all fractions are recognized by the anti-RT monoclonal antibody 1E8. The failure of the antibody fragments to bind to the enzymatically inactive RT form represents a very stringent specificity control and suggests that the antibodies recognize a conformational epitope important for the enzymatic activity. The epitope could be contributed by both monomeric subunits or, alternatively, could be induced on one of the two subunits only upon homo- or heterodimerization.

Reactivity of anti-HIV-1 RT antibody fragments with other RT and DNA polymerase enzymes. The antibody fragments were further tested for their ability to inhibit the RNA-dependent DNA polymerase activity of RT enzymes from other, unrelated retroviruses, such as Moloney murine leukemia virus and avian myeloblastosis leukemia virus. The antibody fragments C7 and D7 neutralize the enzymatic activity of these enzymes as well (Fig. 4B and C). This is particularly noteworthy in view of the fact that these enzymes show little sequence homology with the HIV-1 RT, thus confirming that a structural epitope, common to these enzymes from distinct origins, is being recognized by the antibody fragments. In view of this result and of the fact that there is structural homology between the domain organizations of HIV-1 RT and of the Klenow fragment of *E. coli* DNA polymerase I (19, 25, 32), the antibody fragments C7 and D7 were tested in a random-primed DNA polymerization catalyzed by the Klenow fragment (Fig. 4D), T7 DNA polymerase (Fig. 4E), or *Taq* polymerase (not shown). Interestingly, the antibody fragments inhibit the DNA polymerase activities of these three enzymes as well, unlike what was observed with terminal deoxynucleotidyl transferase (Fig. 4F). In this case, the antibody fragments failed to inhibit this DNA polymerase, which, in contrast to the other polymerases, is template independent. Prompted by these results, we asked whether the antibody fragments could exert their inhibitory action on the mammalian DNA pol α and β as well. The enzymatic activities of the two mammalian enzymes were assessed in parallel for pol α and pol β, using a poly(dA)-oligo(dT) template primer. The results (Fig. 5) show that while the selected antibody fragments inhibit pol α, they are totally ineffective against pol β. This is particularly intriguing in view of the fact that pol β has been suggested to belong to a distinct family of DNA polymerases and to be more similar to deoxynucleotidyl transferase than to the prokaryotic DNA polymerases Klenow, *Taq*, and T7 (7, 16, 18, 25).

In conclusion, these results suggest that the selected antibody fragments recognize a structural and functionally important epitope on HIV-1 RT, which is shared with template-dependent replicative DNA polymerases.

DISCUSSION

Current efforts to block HIV-1 replication focus on finding chemical compounds that selectively interfere with essential steps of the viral life cycle and replication without significantly affecting the host cells. One major target for HIV chemotherapy is the RT protein, produced by proteolytic processing from the polyprotein gene product of the *gag-pol* gene complex. Although there has been substantial progress in blocking HIV-1 replication in vitro, problems arise from the emergence of viral strains with point mutations in the RT-coding region, which prevent these drugs from inhibiting the retroviral enzyme (for a review, see reference 1). This has been an impetus for the exploration of genetic approaches to interfere with HIV-1 infection. One recently developed experimental strategy that holds great potential for schemes for gene therapy for HIV is represented by antibody-mediated intracellular immunization (4; for reviews, see references 3 and 29). This approach is based on the ectopic expression of recombinant forms of antibodies targeted to particular cellular compartments to interfere in a specific manner with the corresponding antigen (3). The basic requirement for this approach is the availability of recombinant forms of a high-affinity neutralizing

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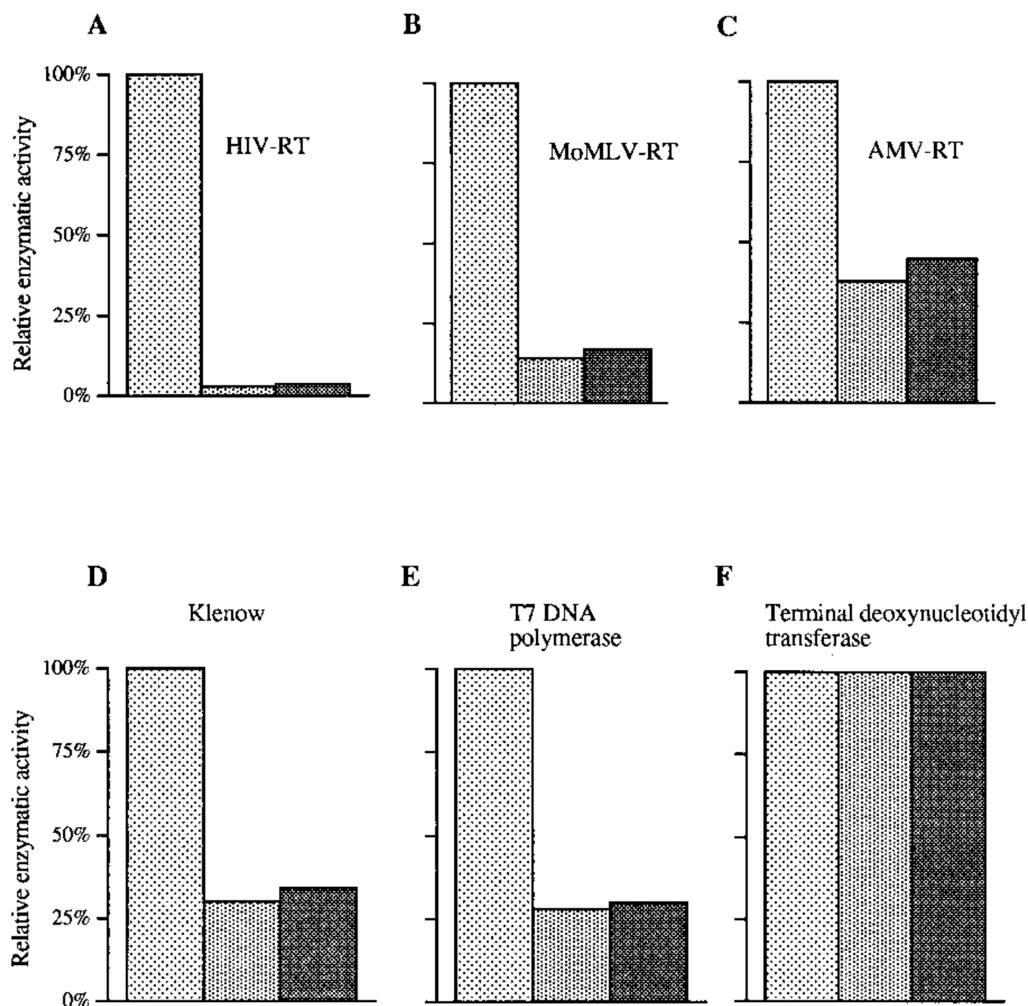

FIG. 4. Effect of anti-HIV-1 RT antibody fragments C7 (▨) and D7 (▩) and a control Fab (▧) on the enzymatic activities of different DNA polymerases. (A) HIV-1 RT (4 nM); (B) mouse Moloney leukemia virus RT (MoMLV-RT) (500 U/ml); (C) avian myeloblastosis leukemia virus RT (AMV-RT) (250 U/ml); (D) Klenow fragment of *E. coli* DNA polymerase I (230 U/ml); (E) T7 bacteriophage DNA polymerase (125 U/ml); (F) terminal deoxynucleotidyl transferase (400 U/ml). The substrate was 10 μg of poly(rC)-oligo(dG) per ml for panels A, B, and C; 1.5 μg of lambda phage DNA per ml for panels D and E; and 10 μg of *Pst*I-linearized plasmid DNA (5.5 kb) per ml for panel F. In all cases the error was less than 4%.

antibody specific for the target antigen. Moreover, the prospective use of intracellular antibodies in a gene therapy perspective requires the antibodies to be of human origin, in order to limit host immune responses.

A number of groups have described mouse monoclonal antibodies prepared against HIV-1 RT (10, 11, 28, 35, 38), but none of these antibodies is recombinant or human. For these reasons, we decided to undertake the isolation of a new antibody against HIV-1 RT that would not suffer from these limitations, by exploiting phage technology and libraries of human antibody fragments displayed on the surface of filamentous phage (37). Besides providing antibodies that are by definition both human and recombinant, this technology has the additional advantage that it bypasses the immune system and thus allows the isolation of antibodies directed against immunologically conserved epitopes, such as the active sites of enzymes. As a source of human recombinant antibodies we used a 2lox-P library of Fab antibody fragments displayed on the surface of filamentous phage (14), which has a diversity of 10^{12}. Of the different antibody fragments isolated after repetitive cycles of panning on solid-phase-coupled purified HIV-1 RT, two were selected for further study. These antibody fragments, which neutralize the RNA-dependent DNA polymerase activity of HIV-1 RT at nanomolar concentrations, display several interesting properties. Both bind only to the enzymatically active form of the RT and do not bind to the inactive p51 or p66 subunit alone, pointing to a delicate structural requirement for the antibody recognition. Their epitope could be contributed by both monomeric subunits or, alternatively, could be induced on one of the two subunits upon homo- or heterodimerization, yielding the active form of the enzyme. The conformational nature of the epitope is confirmed by the failure of the antibody fragments to bind to any linear peptides derived from RT. This epitope appears to be important for the RNA-dependent DNA polymerase activity of the protein, as also confirmed by the fact that the antibodies neutralize the enzymatic activities of (i) RTs from unrelated retroviruses, (ii) DNA polymerase enzymes of prokaryotic origin, and (iii) human DNA pol α. All of these polymerase proteins show very little sequence homology to the HIV-1 enzyme, but, as shown by very recent structural studies (5, 13, 18, 19, 25), all display a similar structure of the palm domain near the polymerase

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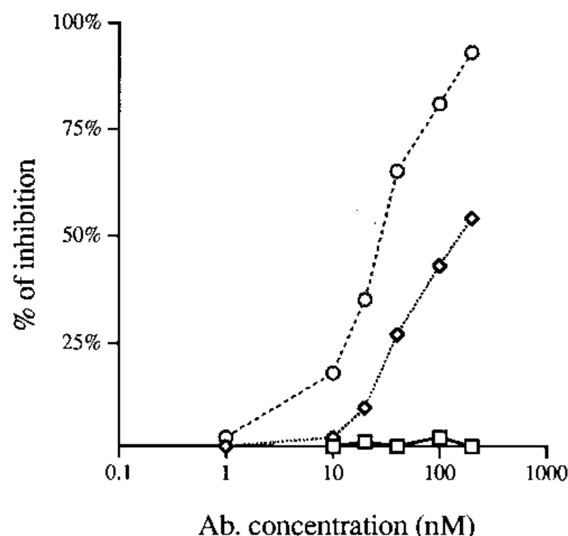

FIG. 5. Effect of anti-HIV-1 RT antibody fragment D7 on the activities of mammalian DNA pol α and pol β. The DNA-dependent DNA polymerase activities of human DNA pol α (30 nM) (◇) and rat DNA pol β (30 nM) (□) were assayed as a function of the concentration of antibody fragment D7 (Ab.), using poly(dA)-oligo(dT) (10 μg/ml) as a substrate. The RNA-dependent DNA polymerase activity of HIV-1 RT (15 nM) (○) is reported for comparison.

active site (reviewed in reference 16), which underlies evolutionary relationships and a common polymerase mechanism (32). Interestingly, the antibody fragments also inhibit the mammalian DNA pol α but are ineffective against DNA pol β. The latter enzyme is not a replicative polymerase but rather is a distributive polymerase and has been suggested to be more similar to the nucleotidyl-transferring enzyme terminal deoxynucleotidyl transferase (7, 25). Interestingly, terminal deoxynucleotidyl transferase is not inhibited by the antibody fragments described here. Thus, the antibody fragments described in this paper appear to recognize a structural fold that is common to different polymerase enzymes and provide an immunological experimental basis for a structural classification of this enzyme family. The epitope recognized is necessary for their enzymatic activity, a conclusion that is strengthened by the observed competitive inhibition with respect to the nucleic acid substrate. The inhibition of RTs from avian and murine retroviruses is particularly noteworthy. Indeed, several of the amino acid mutations that induce drug resistance in HIV-1 RT (1) are found in Moloney murine leukemia virus RT as the native sequence, for example, Leu-74 to Val and Met-184 to Val, which confer resistance of HIV-1 RT to dideoxyinosine and dideoxycytidine, respectively (13). Thus, the tolerance of the antibody domains described in this paper to amino acid changes between different polymerases that preserve the polymerase activity makes them prospects for reagents suitable for the inhibition of viral escape RT mutants as well.

The inhibitory activity of these antibody fragments towards DNA polymerase could represent a source for concern, in view of their prospective utilization for intracellular expression. However, providing the antibody with suitable targeting signals should ensure that the antibody does not interfere with cellular polymerases, and indeed, stable human lymphoblastoid cell lines and murine fibroblast cells expressing the cytosolic forms of these antibody domains show a normal growth rate (data not shown). In these stably transfected cells, efficient inhibition of viral retrotranscription can be demonstrated (12a).

These antibody fragments could represent the starting point not only for their use in a gene therapy perspective but also for the design and selection of improved antibody domains and of drugs inhibiting both the native HIV-1 RT and mutant variants thereof. The experimental determination of the combining site between these antibody domains and RT will be of great help towards attaining the goal of achieving a structure-based design of new anti-HIV RT drugs (12).

In conclusion, the antibody fragments described in this paper represent structural probes for the active sites of RT and other members of the DNA polymerase family. The results obtained provide experimental evidence for the evolutionary structural relatedness of these enzymes.

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