A novel neutralization epitope on the 'thumb' subdomain of human immunodeficiency virus type 1 reverse transcriptase revealed by a monoclonal antibody

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We have prepared a MAb, 7C4, which inhibits the RNA-dependent DNA polymerase activity of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT); this MAb has allowed identification of a previously unknown neutralizing epitope of RT. Analysis of the epitope and of the mechanism of polymerase inhibition revealed that 7C4 acts by interfering with the interaction between RT and the template–primer. 7C4 recognizes a discontinuous epitope on the two α-helices, αH and αI, that make up the ‘thumb’ subdomain of RT. The existing crystallographic model of HIV-1 RT suggests that the ‘thumb’ subdomain, together with the ‘fingers’ and ‘palm’, form a nucleic-acid-binding cleft in the 66 kDa subunit of RT and that αH is in contact with the primer strand of the template–primer. The extent of inhibition of enzyme activity produced by 7C4 correlates with the reported primer-length-dependency of template–primer binding to RT. Inhibition by 7C4 was competitive with respect to the template–primer and mixed with respect to the substrate. Binding of 7C4 to RT was prevented by preincubation of the enzyme with high concentrations of template–primer but not with substrate. Thus, the 7C4 epitope apparently exists on part of the template–primer binding site of the αH and αI regions of the ‘thumb’ subdomain. This neutralization epitope is a logical target for the development of new types of HIV-1 RT inhibitors.

Introduction

The pivotal role played by reverse transcriptase (RT) in the replication of retroviruses has made this enzyme a key target for chemotherapy of human immunodeficiency virus type 1 (HIV-1) infection (Mitsuya et al., 1990; Larder, 1993). The most promising antiviral agents known to inhibit the function of HIV-1 RT fall into two broad classes: chain-terminating nucleoside analogues such as 3'-azido-3'-deoxythymidine (AZT) and non-nucleosides that interact directly with RT (De Clercq, 1992). However, cytotoxicity problems (especially with nucleoside analogues) together with the emergence of drug-resistant variants of the virus have compromised the effectiveness of these drugs (Larder, 1993, 1994). Development of more potent and less cytotoxic inhibitors requires detailed knowledge of the functionally important sites of HIV-1 RT as well as of the mechanisms of inhibition by these drugs and of resistance to them.

Recent crystallographic studies (Kohlstaedt et al., 1992, 1993; Jacobo-Molina et al., 1993; Smerdon et al., 1994; Rodgers et al., 1995; Ren et al., 1995; Esnouf et al., 1995; Ding et al., 1995) have provided considerable information on the structure of HIV-1 RT: the organization of motifs in the active site, the role of each subdomain of the enzyme in catalytic cycling, and the targets for both classes of inhibitors. HIV-1 RT functions as a heterodimer composed of a 66 kDa and a 51 kDa subunit (p66 and p51, respectively). The p66 subunit folds into two distinct domains: a typical polymerase domain and a connected ribonuclease (RNase) H domain. The second subunit, p51, is a copy of p66 in which the 15 kDa RNase H carboxy-terminal segment has been cleaved by the HIV-1 protease; it lacks a functional nucleic acid binding cleft. The polymerase domain, p66, consists of four subdomains, referred to as...
'fingers', 'palm', 'thumb' and 'connection' by analogy with the parts of the right hand (Kohlstaedt et al., 1992). The target for the nucleoside inhibitors is the substrate binding site on the 'palm' subdomain and the target for the non-nucleoside inhibitors is the polymerase active site on the 'palm' subdomain.

Many groups have successfully produced monoclonal antibodies (MAbs) to HIV-1 RT, since some MAbs clearly reveal protein structure–function relationships (Harris, 1984; Friguet et al., 1989). Although most of these MAbs have been ineffective in neutralizing the polymerase activities of RT (Hansen et al., 1988; Tisdale et al., 1988; Ferris et al., 1990; Li et al., 1993), a few inhibitory MAbs have been reported (Ferns et al., 1991; Örvell et al., 1991; Szilvay et al., 1992; Restle et al., 1992; Wu et al., 1993), and these may point to functionally important sites of RT. For example, MAb 28 (Restle et al., 1992) and MAb 1E8 (Wu et al., 1993) are involved in blocking binding of deoxynucleoside triphosphate substrate to the enzyme. Although AZT and other chain-terminating nucleoside analogues were discovered by screening for inhibitory activity against HIV-1 replication in culture, it later became evident that they interact with the substrate binding sites recognized by the MAbs. Thus, neutralizing MAbs show promise as powerful alternative tools to identify functionally important sites on the enzyme, and thus for developing new inhibitors of its function.

We have prepared a MAb, 7C4, which can almost completely inhibit the RNA-dependent DNA polymerase activity of HIV-1 RT. In the present paper, we analysed the epitope on RT that it recognizes and the mechanism by which enzyme activity is inhibited. The utility of a novel neutralizing epitope recognized by 7C4 on the 'thumb' subdomain of HIV-1 RT in the search for new types of RT inhibitors is discussed.

Methods

- **Preparation of antibodies.** A hybridoma line producing MAb 7C4 (IgGl, k) was established in a cell fusion experiment using spleen cells from a mouse repeatedly infected with a vaccinia virus recombinant that contained the RT gene of HIV-1 (HTLV-IIIB strain) (Hoshikawa et al., 1991). Culture supernatants containing MAb 7C4 inhibit RNA-dependent DNA polymerase activity both of HIV-1 virions and of recombinant HIV-1 RT expressed in E. coli (T. Kobayashi, J. Chiba, A. Yasuda, A. Kojima & T. Kurata, unpublished). MAb 7C4 was purified from culture supernatants or SCID mouse ascitic fluid by precipitation with (NH₄)₂SO₄ followed by high-performance liquid chromatography on a hydroxyapatite column (Yamakawa & Chiba, 1988) and affinity chromatography using a protein A column (Ampure PA-kit; Amersham). Fab fragments of 7C4 were prepared using immobilized papain on agarose beads (Pierce) according to the manufacturer's instructions. Undigested 7C4 and its Fc fragments in the reaction mixture were removed by protein A affinity chromatography.

- **Preparation of HIV-1 RT.** Recombinant HIV-1NL4-3 RT was expressed in E. coli and purified as described by Satoh et al. (1990). The expression system allows production of large quantities of heterodimeric p51/p66 RT correctly processed by the HIV-1 protease. The purified RT typically has a specific activity of 10000–20000 units/mg, where 1 unit is the amount of enzyme that incorporates 1 nmol of [³H]TMP into poly(rA)·oligo(dT)₁₀ in the assay for RNA-dependent DNA polymerase.

- **Determination of the 7C4 epitope on HIV-1 RT.** To map the 7C4 epitope, the reactivity of 7C4 was first tested with the various segments of RT protein expressed in E. coli as fusion proteins with β-galactosidase, and then with synthetic polypeptides of defined sequences from the HIV-1 pol gene. Descriptions of the plasmids used to express modified segments of HIV-1 RT will be published elsewhere (A. Saito & H. Shimagawa). Reactivities of fusion proteins with MAb 7C4 were tested either by Western blot analysis after SDS–PAGE of cell lysates or dot blot analysis without treatment with SDS. To fine-map the binding site for 7C4, antibody-reactive peptide scanning was carried out using a set of overlapping synthetic octapeptides, either by the multiple peptide synthesis technique (Chiron Mimotopes) developed by Gysen et al. (1987) or the usual ELISA method with peptides produced by Merrifield solid-phase synthesis and purified by reverse-phase chromatography.

- **RT enzymatic activity assay.** RT polymerase activity was assayed essentially as described by Hoffman et al. (1985). Unless otherwise indicated, the RNA-dependent DNA polymerase activity of HIV-1 RT was measured in a reaction mixture containing 50 mM-Tris–HCl, pH 8·0, 5 mM-dithiothreitol, 5 mM-MgCl₂, 150 mM-KCl, 0·3 mM-glutathione (reduced form), 0·5 mM-EGTA; 0·06% Triton X-100, 6 mM-[³H]thymidine triphosphate (Du Pont), 5 µM-poly(rA)·oligo(dT)₁₀ (Pharmacia), 0·2 mg/ml BSA and 0·5 mg/ml RT. After incubation at 37 °C for 30 min, the mixture was spotted onto Whatman filter paper DE81, washed, and radioactivity was measured in a liquid scintillation counter. In this study, poly(rA)·oligo(dT)₁₀ and TTP were used as the template–primer and substrate, respectively, since poly(rA)·oligo(dT)₁₀ is the most efficient primed template for the p66.p51 heterodimer (Thimmig & McHenry, 1993). The DNA-dependent DNA polymerase activity of HIV-1 RT was assayed in a similar reaction medium (Thimmig & McHenry, 1993).

- **Kinetics of inhibition by MAb 7C4 of RNA-dependent DNA polymerase activity.** The effects on the reaction rate of incubation of RT with 7C4 prior to and at the same time as initiation of the DNA synthesis reaction were compared. Preincubation of RT in the presence or absence of 7C4 was carried out in Buffer A: 50 mM-Tris–HCl, pH 7·4 containing 100 mM-NaCl, 5 mM-EDTA and 0·5 mg/ml BSA. The mechanism of 7C4-mediated inhibition was analysed by using steady-state kinetics and varying both the template–primer or the substrate, and the antibody concentrations.

Template–primer effects on 7C4 binding to RT. The ability of template–primer to prevent binding of 7C4 to RT was analysed by two different methods: its effect on RT binding to 7C4 immobilized either on Sepharose beads (Pharmacia) or a dextran-coated sensor chip (Pharmacia). In the Sepharose beads experiment, the amount of RT bound to 7C4 was determined by measuring residual RT activity in the supernatant after centrifugation of the reaction mixture. In the latter experiment, molecular interaction between 7C4 and RT was directly monitored using a BIAcore instrument (Fagerström, 1991; Johnson et al., 1991), which is capable of detecting binding of one or more soluble proteins to a protein immobilized on a dextran-coated chip by a difference in refractive index (Stenberg et al., 1991). 7C4 was coupled to the dextran matrix by standard amine chemistry according to the manufacturer's instructions. A flow of HBS (10 mM-HEPES containing 150 mM-NaCl, 3·4 mM-EDTA and 0·005% Surfactant P20, pH 7·4) was maintained over the sensor surface at 5 µl/min. Samples were injected at 5 µl/min for 4 min each. The dissociation reagent was 100 mM-glycine–HCl buffer, pH 3·5, containing 2 mM-NaCl. Between injections, the surface was regenerated with HBS.
Results

Determination of the epitope on HIV-1 RT recognized by 7C4

7C4 was reactive with both the p51 and p66 subunit of RT in the Western blotting analysis (data not shown). To locate the epitope recognized by 7C4, various peptide segments of RT were expressed in E. coli as fusion proteins with b-galactosidase and tested for reactivity with 7C4 by the Western and dot blotting analyses. Fig. 1 summarizes the location of the segments tested and their reactivity with 7C4. The 7C4 epitope was found to be located on residues within the region spanning amino acids 252–335 in the HIV-1 RT sequence, which contains sequences of the ‘thumb’ subdomain.

In order to map the region more precisely, the epitope was scanned by the PEPSCAN technique (Geysen et al., 1987) using a set of overlapping 8-mer synthetic peptides covering amino acids 230–340. Interestingly, 7C4 reacted with three peptide segments, 260LVGK, 271YPGIKV and 280LRGTKA, distant in the sequence (Fig. 2a). These are segments of a-helix H, a peptide between a-helices H and I, and part of a-helix I, respectively, which make up the thumb subdomain (Jacobo-Molina et al., 1993). This finding indicates that 7C4 recognizes a discontinuous epitope (tertiary structure) that consists of amino acids in the linear sequence which are brought together by folding. Reactivity of 7C4 with the discontinuous epitope was confirmed by ELISA using a similar set of purified overlapping synthetic octapeptides (Fig. 2b).

Inhibition of RNA-dependent DNA polymerase activity by 7C4

Preincubation of RT with 7C4 resulted in dose-dependent inhibition of RNA-dependent DNA polymerase activity with complete inhibition at approximately equimolar concentrations of RT and 7C4 in the incubation mixture (Fig. 3). Similar results
were obtained when Fab fragments of 7C4 were tested, although a higher concentration (about 40-fold) was required for complete inhibition (Fig. 3). Significant but incomplete (38%) inhibition of DNA-dependent DNA polymerase activity was observed when the enzyme was exposed to the same or double the concentration of 7C4 that caused complete inhibition of RNA-dependent DNA polymerase activity (data not shown).

**Effect of primer length of the template–primer on inhibition of RNA-dependent DNA polymerase activity by 7C4**

Since our preliminary observation indicated that 7C4 may act as a competitive inhibitor with poly(rA)•oligo(dT)12–18 in the steady-state kinetics assay (data not shown) and since binding of poly(rA)•oligo(dT)n to HIV-1 RT is primer-length-dependent (Reardon et al., 1991), we investigated the effect of primer length on inhibition of RNA-dependent DNA polymerase activity by 7C4. Almost complete inhibition (90–99% of control) was observed when the primer length was 12 nucleotides or less (Table 1). Strong but incomplete (71–74%) inhibition was observed when the primer length was 15 or more. Since complete inhibition of the polymerase activity was observed with poly(rA)•oligo(dT)10 and since it has been further reported that the RNase H domain does not contribute to template–primer binding when the primer length is less than 13 nucleotides (Reardon et al., 1991), unless otherwise indicated poly(rA)•oligo(dT)10 was used for all subsequent inhibition assays.

**Table 1. Effect of primer length on 7C4 inhibition of HIV-1 RT RNA-dependent DNA polymerase activity**

HIV-1 RT (0.5 ng/ml) was incubated with the indicated concentrations of 7C4 in Buffer A for 45 min at room temperature. Samples (20 μl) were then added to 30 μl of a solution that produced the appropriate concentration of the reaction components for the polymerase activity assay containing poly(rA)•oligo(dT)n as template–primer and [3H]dTP as deoxynucleoside triphosphate substrate. After an additional 30 min incubation at 37 °C the mixture was spotted onto filter paper to determine dTMP incorporation, as described in Methods. When n was 8, 10, 12, 15 and 18, the amounts of dTMP incorporated by RT with poly(rA)•oligo(dT)n in the absence of 7C4 treatment were 23, 59, 81, 114 and 115 pmol, respectively.

<table>
<thead>
<tr>
<th>Primer length of poly(rA)•oligo(dT)n</th>
<th>RT activity</th>
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<tbody>
<tr>
<td></td>
<td>Antibody concn (μg/ml): 0.03, 0.5, 1.0</td>
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<tr>
<td>n = 8</td>
<td>60, 98, 99</td>
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<tr>
<td>n = 10</td>
<td>54, 96, 97</td>
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<tr>
<td>n = 12</td>
<td>46, 89, 93</td>
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<tr>
<td>n = 15</td>
<td>38, 63, 71</td>
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<tr>
<td>n = 18</td>
<td>36, 63, 74</td>
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* Percentage inhibition of RT activity in the -7C4 control.

**Kinetics of 7C4 inhibition of RNA-dependent DNA polymerase activity**

To examine the mechanism of inhibition of the RNA-dependent DNA polymerase activity by 7C4, the effects on
the reaction rate of incubation with 7C4 prior to and at the same time as initiation of DNA synthesis were compared. In contrast to the complete inhibition observed when RT was preincubated with 7C4, only slight inhibition of enzyme activity occurred when 7C4 was added to the reaction mixture at the same time as RT (Fig. 4). No effect on the reaction rate of the enzyme was observed when an unrelated MAb (anti-human serum albumin; 3E7) was added to the reaction mixture (Fig. 4). These findings indicate that 7C4 specifically inhibits polymerase activity by binding to the free enzyme.

To obtain more detailed information on its mode of action, the mechanism of 7C4-mediated inhibition was analysed by using steady-state kinetics and varying both the template–primer, or the substrate, and the antibody concentrations. In the presence of constant concentrations of TTP, the double reciprocal plots of polymerase activity versus poly(rA)· oligo(dT) concentration were linear at all antibody concentrations and inhibition appeared to be competitive (Fig. 5a). In contrast, the plots of enzyme activity versus TTP concentration in the presence of a constant poly(rA)· oligo(dT) concentration yielded a mixed-type inhibition pattern (Fig. 5b). The competitive pattern of inhibition indicates that template–primer and 7C4 act on the same conformation of the enzyme, probably near the same site.

**Ability of template–primer to prevent binding of 7C4 to RT**

Steady-state kinetic data for the enzyme may not allow the correct interpretation of the kinetic mechanism of inhibition since the polymerization reaction comprises several steps (Majumdar et al., 1988; Reardon, 1992). In order to investigate directly the possibility that the binding site for 7C4 is the same as, or very close to that involved in template–primer...
interactions, the ability of poly(rA)• oligo(dT) to prevent binding of 7C4 to RT was assessed. When RT was preincubated with different concentrations of poly(rA)• oligo(dT)$_{10}$, then reacted with immobilized 7C4 on agarose beads, a dose-dependent increase in the amount of residual activity in the supernatants (that is, decrease in the amount of RT bound to immobilized 7C4) was noted (Fig. 6). Little, if any, effect of preincubation with poly(rA)• oligo(dT)$_{10}$ on binding of RT to a non-inhibitory anti-RT MAb (3D12) was observed; the activity in the reaction medium was almost completely absorbed by immobilized 3D12 (Fig. 6). TTP did not affect the interaction between 7C4 and RT (data not shown). Similar results were obtained when the molecular interaction of 7C4 and RT was monitored using the BIAcore instrument. Preincubation of RT with various concentrations of poly(rA)• oligo(dT)$_{10}$ resulted in dose-dependent inhibition of binding of RT to 7C4 immobilized on the sensor chip (Fig. 7a). Again, TTP did not affect the interaction between 7C4 and RT (Fig. 7b).

Discussion

In the present study, we have extensively characterized a murine MAb, 7C4, that strongly inhibits the RNA-dependent DNA polymerase activity of HIV-1 RT. The results clearly indicate that 7C4 is reactive with a novel neutralizing epitope of RT and acts by interfering with the interaction between RT and the template–primer. This conclusion is based on having demonstrated (1) that the 7C4 epitope is located on the ‘thumb’ subdomain of the polymerase domain postulated to be engaged in template–primer binding, (2) that 7C4 mediates specific and strong inhibition of polymerase activity by binding to the free enzyme, (3) that inhibition mediated by Fab fragments of 7C4 is competitive with respect to the template–primer, (4) that inhibition depends on the primer length of the template–primer and (5) that protection from 7C4 binding to RT is achieved only by preincubation of RT with higher concentrations of the template–primer, not the substrate.

It should be noted that the extent of inhibition produced by 7C4 (Table 1) closely correlated with the reported (Reardon et al., 1991) primer-length-dependency of poly(rA)• oligo(dT)$_{n}$ binding to HIV-1 RT. 7C4 inhibited the activity of HIV-1 RT almost completely (93–99% of control) when the primer length of the template–primer was 12 nucleotides or less. Strong but incomplete (71–74%) inhibition was observed when the primer length was 15 or more. Because there is an abrupt transition at a primer length between 14 and 16 nucleotides in the $K_d$ for binding of poly(rA)• oligo(dT)$_{n}$ to RT (Reardon et al., 1991), these results strongly indicate that inhibition of polymerase activity by 7C4 is due to competition with the template–primer and that the extent of inhibition depends on the affinity of 7C4 and the template–primer for RT.

Dose-dependent inhibition of binding of 7C4 to RT by the template–primer as demonstrated by two different methods (Figs 5 and 6) supports these hypotheses. Determination of the $K_d$ for binding of RT to 7C4 and quantitative analysis of the interaction between RT and 7C4 and poly(rA)• oligo(dT)$_{n}$ with the BIAcore instrument have also provided supporting evidence (unpublished).

The epitope identified by 7C4 appears to be discontinuous or conformational in nature, and has been located on three non-contiguous peptide segments: $^{260}$LVGK, $^{271}$YPGIKV and $^{283}$LRGTKA (Fig. 2). These segments are, respectively, a part of a-helix H, a peptide between a-helices H and I, and part of a-helix I, which make up the ‘thumb’ subdomain (Jacobo-Molina et al., 1993). Our analysis by computer algorithms (Janin, 1979; Hopp & Woods, 1981) has shown that the segments residing within the antigenic epitope recognized by 7C4 are probably located on the surface of RT (unpublished data). Our findings are more consistent with the aH and aI regions of p66 interacting with the template and/or primer strand (Jacobo-Molina et al., 1993). The ‘thumb’ subdomain of p66 is postulated to be engaged in template–primer binding and the two helices are suspected of functioning as ‘tracks’ on
which the template–primer moves during translocation (Jacobo-Molina et al., 1993) or of acting as a kind of ‘helix clamp’ (Hermann et al., 1994). A recent report on analysis, using alanine scanning mutagenesis, of the structure–function relationship of the thumb subdomain (Beard et al., 1994) has suggested that the α1 core (residues Gln-258, Gly-262 and Trp-266) interacts with the template–primer. 7C4 very likely binds to a part of this core region, resulting in blocking of template–primer binding and a subsequent change in orientation of the p66 ‘thumb’ (Rodgers et al., 1995). At present, it is unclear whether 7C4 simultaneously binds to another epitope that may be present on the p51 thumb subdomain and affects other enzyme activities. We did not examine the effect of 7C4 on the RNase H activity of HIV-1 RT in this study, since the 7C4 epitope was found to be located exclusively on the polymerase domain. However, the possibility remains that 7C4 affects RNase H activity sterically or allosterically through interaction with the ‘thumb’ subdomain. Co-crystallization experiments using Fab fragments of 7C4 and RT complexes will reveal which amino acid residues on each molecule are involved in the interaction. Such experiments may also elucidate the following points concerning the enzyme: (1) why 7C4 inhibits the activity of RNA-dependent DNA polymerase more strongly than DNA-dependent DNA polymerase, (2) how conformational changes in RT occur during translocation, (3) whether 7C4 binds to the ‘thumb’ subdomain of the p51 subunit, and (4) whether 7C4 affects structure and function of the RNase H.

Part of the 7C4 epitope seems to be located on a highly conserved region of retroviral pol genes: Region F described by Larder et al. (1987) or Region 8 described by Jacobo-Molina & Arnold (1991). Region F and Region 8 can be aligned with a region of the primary sequence from E. coli DNA polymerase I implicated in dNTP binding (Basu et al., 1989). However, 7C4 seems to be specific for HIV-1 among retroviruses. Our preliminary findings (unpublished) suggest that 7C4 inhibits RT activity in the culture supernatant of human T cell lines producing three strains of HIV-1 (IIIB, Bru and IMS-1) but not of cell lines producing two strains of HIV-2 (GH-1 and LAV-2) or two strains of SIV (MAC and MND). In addition, no significant inhibition of RNA-dependent DNA polymerase activity of RT from HIV-2 or SIV by 7C4 has been observed; nor has any reactivity of 7C4 against the polymerase domain. However, the possibility remains that 7C4 affects RNA polymerase activity sterically or allosterically through interaction with the ‘palm’ subdomain. Co-crystallization experiments using Fab fragments of 7C4 and RT complexes will reveal which amino acid residues on each molecule are involved in the interaction. Such experiments may also elucidate the following points concerning the enzyme: (1) why 7C4 inhibits the activity of RNA-dependent DNA polymerase more strongly than DNA-dependent DNA polymerase, (2) how conformational changes in RT occur during translocation, (3) whether 7C4 binds to the ‘thumb’ subdomain of the p51 subunit, and (4) whether 7C4 affects structure and function of the RNase H.

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