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Inhibition of Heat Inactivation of Reverse Transcriptase of Human Immunodeficiency Virus Type 1 by Seropositive Sera

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The reverse transcriptase (RT) activity of a lysate of human immunodeficiency virus type 1 (HIV) was almost completely inactivated by incubation at 56°C for 20–30 min. The heat-inactivation of RT in the virus lysate or purified RT was partially inhibited in the presence of some human sera or plasma containing antibodies against HIV. The IgG fraction purified from the seropositive sera was responsible for stabilization of RT upon heat inactivation. This is a new assay system for detection of antibodies against RT.

Key words: HIV-1 — Reverse transcriptase — AIDS — Antibody

Human immunodeficiency virus type 1 (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS).[1-4] Infection with HIV results in production of antibodies against viral proteins: antibodies against the internal core (*gag*) proteins and the envelope (*env*) proteins have been found.[5-10] The viral proteins that react well with HIV-infected human sera are p19 and p25 *gag* proteins, Pr55 *gag* precursor protein, and gp160, gp120 and gp41 *env* proteins. Recently, antibodies against reverse transcriptase (RT) of HIV have also been demonstrated by Western blot assay or neutralization assay.[11-14] p66/p51 or p64/p53, detected by Western blot assay, was found to be derived from the *pol* gene product.[7, 14] IgGs purified from the sera of seropositive subjects were shown to neutralize the RT activity.[13, 14] Furthermore, inverse relationships were found between the severity of HIV infection and both the titers of antibodies that neutralized RT[13] and the titers of those against core *gag* proteins.[15, 16] We tried to develop a new assay system to detect antibodies against RT in a small volume of whole human serum or plasma. RT of human T-cell leukemia virus type 1 (HTLV-1) is inactivated by heat-treatment at 56°C for 20–30 min, as described.[17] RT of HIV is also inactivated by this heat-treatment. We thought that the inactivation of RT by heat-treatment might be due to destruction of its tertial structure and that this inactivation might be partially inhibited by antibodies bound to specific regions of RT. This paper reports studies suggesting that this is, in fact, the case.

MATERIALS AND METHODS

Sera and plasma Human sera or plasma were obtained from normal subjects, HTLV-1 antibody-positive healthy adults, patients with adult T-cell leukemia (ATL), HIV antibody-positive asymptomatic carriers (AC) and patients with AIDS and AIDS-related complex (ARC). Seropositive patients infected with HIV-1 were classified by CDC criteria: AC represents group II and ARC includes group III and group IV (subgroup A). HIV antibody-positive plasma and sera were mainly obtained from Japanese hemophiliacs and British homosexuals, respectively. They were heated at 56°C for 30 min before use.

Virus lysates HIV was prepared from the culture fluids of MOLT-4 cells[18] and U937 cells[19] infected with HTLV-III$_B$ and HIV[GUN-1],[4, 20] respectively. HTLV-1, bovine leukemia virus (BLV), and Moloney murine leukemia virus (MoMuLV) were obtained from culture fluids of MT-2, FLK and YAC-1 cells, respectively, as described elsewhere.[21] Culture fluids containing retroviruses were centrifuged for 15 min at 2,500 rpm. The supernatants (75 ml) were mixed with 36 ml of 30% polyethylene glycol (PEG) and 3 ml of 4 *M* NaCl, stood on ice for 2 h and centrifuged for 30 min at 2,500 rpm at 0°C. The precipitates were suspended in 2 ml of suspension buffer consisting of 25 m*M* Tris-HCl (pH 7.5), 50 m*M* KCl, 50% glycerol, 0.025% Triton-X 100 and 5 m*M* dithiothreitol and 1 ml of lysis buffer consisting of 1.5 *M* KCl and 0.9% Triton-X 100. Virus lysates were stored at −80°C. Virus lysates gave similar RT activities when prepared and assayed on different occasions.

RT assay The virus lysates prepared as described above were used for RT assay, which was conducted essentially as described elsewhere.[21, 22] Briefly, virus lysates (20 μl) were mixed with 2 μl of TNE buffer [20 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl and 1 m*M* EDTA], human sera or plasma, human IgG or mouse monoclonal antibody and incubated first at 37°C for 10 min and then at 37°C or 56°C for 20 min. The RT reaction mixture (50 μl) was then added to the virus lysates. This RT reaction mixture contained poly(rA) and oligo(dT)$_{12-18}$ as template and

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primer and [^{3}H]TTP as substrate, as described.[21,22] The mixture was incubated at 37°C for 1 h, and then spotted onto DE-81 filters, which were washed with 0.5 *M* Na_2HPO_4. The radioactivity bound to the filters was counted in a liquid scintillation counter.

Purification of RT The RTs of HTLV-III$_B$ and HIV-[GUN-1] virus were purified from the culture fluids of MOLT-4/HTLV-III$_B$ cells and U937/GUN-1 cells, respectively. For this, the culture fluids were centrifuged at 80,000*g* for 1.5 h and the precipitates were suspended in TNE buffer. The virus suspensions were disrupted with lysis buffer [50 m*M* Tris-HCl (pH 7.5), 1 m*M* dithiothreitol, 0.1 m*M* EDTA, 0.3% Nonidet P-40 and 10% glycerol] and subjected to successive column chromatographies on DEAE-cellulose and phosphocellulose as described.[23] Fractions that showed high RT activity were pooled and concentrated in a Minicon-5 (Amicon, MA). The partially purified RT fractions were electrophoresed in SDS-polyacrylamide gels and subjected to silver staining. Two major bands corresponding to p66 and p51 were detected.

Purification of human IgG IgG fractions were purified from sera of normal and HIV-seropositive subjects and an ATL patient using Affigel-Protein A (Bio-Rad, CA) by the method recommended by the supplier. HIV-seropositive sera were obtained from a hemophiliac with ARC and an AC hemophiliac. Pooled IgG fractions were concentrated to one-fifth of their original serum volume in a Minicon-5.

Absorption of sera with Pansorbin cells One volume of normal serum or HIV-seropositive human serum was mixed with 1/10 volume of Pansorbin cells (Calbiochem, CA), namely protein A-coated cells, and then incubated at room temperature for 30 min and centrifuged. The supernatants were treated twice more with Pansorbin cells in the same way to deplete them of IgG.

Immunofluorescence assay MOLT-4/HTLV-III$_B$ cells were smeared onto slide glasses and fixed with acetone. Antibody against HIV was detected by indirect immunofluorescence assay (IF) as described elsewhere.[20] Antibody titers are given as the reciprocals of the highest serum or plasma dilutions exhibiting a positive reaction.

Stabilization and neutralization of RT activity by human sera Time course studies showed that the RT activities of lysates of HIV, HTLV-1, BLV and MoMuLV were almost completely lost on heat-treatment at 56°C for 20 min. As described in this paper, heat inactivation of the RT of HIV at 56°C was partially inhibited in the presence of some human sera or plasma positive for HIV antibody. The inhibitory effects of human serum or plasma on heat inactivation of HIV RT were expressed numerically, as stabilizing indices, determined as follows. Four replicate samples of virus lysate (20 μl) were mixed with human sera or plasma (2 μl) and incubated for 10 min at 37°C. Then two of the samples were incubated at 37°C for another 20 min, while the other two were incubated at 56°C for 20 min. Then 50 μl of RT reaction mixture was added to each sample and remaining RT activity was measured as described above. The stabilizing or neutralizing index of each sample was calculated as follows: Stabilizing index (%) = (RT activity (cpm) after treatment at 37°C for 10 min and then at 56°C for 20 min/RT activity (cpm) after treatment at 37°C for 30 min) × 100. Neutralizing index (%) = (1 − RT activity (cpm) after incubation of virus lysate at 37°C for 30 min in the presence of human serum or IgG/RT activity (cpm) after incubation of virus lysate at 37°C for 30 min in the absence of human serum or IgG) × 100.

RESULTS

Heat inactivation of RT HIV lysates (20 μl) containing RT were incubated for up to 30 min at 37°C or 56°C in the presence of 2 μl, namely 10%, of normal human serum or serum of an ARC patient containing antibody against HIV (Fig. 1). Then residual RT activities were measured. HIV RT was almost completely inactivated by

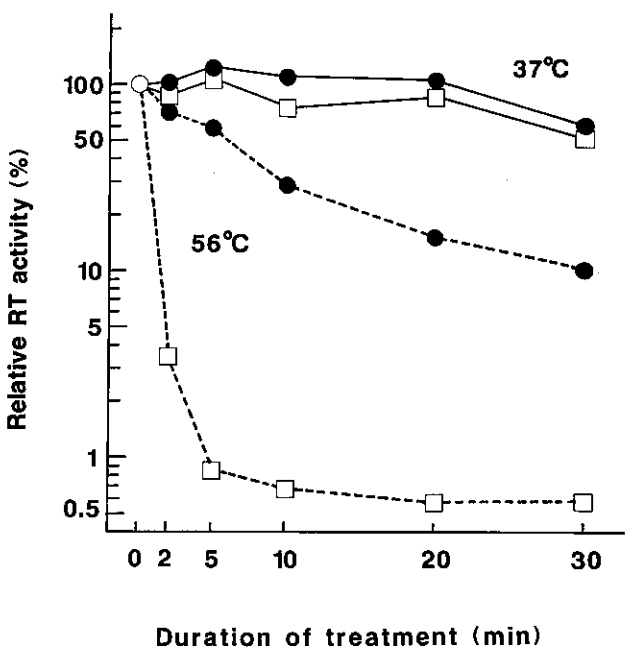

Fig. 1. Time courses of heat inactivation of HIV RT. HIV lysates (20 μl) prepared by PEG treatment were incubated for 0–30 min at 37°C (solid lines) or 56°C (broken lines) in the presence of serum (2 μl) of a normal subject (□) or an ARC patient (●). Then residual RT activities were assayed. The activities of samples without heat-treatment were about 1×10^5 cpm.

heating at 56°C for 10–20 min in the presence of normal human serum, but was scarcely affected by incubation at 37°C for up to 30 min. Inactivation of RT of HIV at 56°C was, however, not complete in the presence of HIV-seropositive serum (Fig. 1). The effect of the serum concentration on heat inactivation of HIV RT was examined. The serum from this ARC patient partially inhibited heat inactivation of HIV RT even at a serum concentration of 2.5%. Namely, residual RT activities were 23% and 4% in the presence of the ARC serum at concentrations of 10% and 2.5%, respectively, whereas they were less than 1% in the presence of the normal human serum at concentrations up to 10%. Based on the above results we examined whether HIV-seropositive sera or plasma had a specific inhibitory effect on heat inactivation of RT.

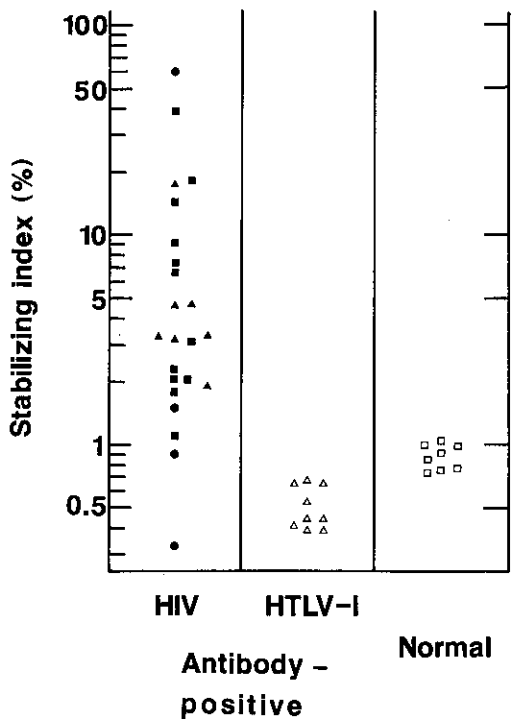

Fig. 2. Relationship between stabilizing indices and neutralizing activities of human sera or plasma. The RT activity detected after reaction in the absence of human serum was taken as 0% neutralizing activity. Symbols: AIDS (●), ARC (▲), AC (■), HTLV-1 antibody-positive (△) and normal subjects (□).

Effects of human sera or plasma on HIV RT First, we tested the sera or plasma for antibodies that would inhibit the RT activity of HTLV-III$_B$. Neutralization assays were done by incubating HIV lysates (20 μl) with human sera or plasma (2 μl) for 30 min at 37°C and then titrating the residual RT activities.

Sera or plasma of 23 HIV antibody-positive subjects obtained from three facilities gave various values. Some samples (about 20%) reduced the RT activity to less than 20% of the serum-free control value; namely, more than 80% inhibition, whereas others gave values within the ranges of those of HIV antibody-negative samples. One serum from an AC hemophiliac strongly inhibited the RT activity (about 99%). This serum was excluded for stabilization analysis because the denominator of the stabilizing index was too small. Thus, the presence of antibodies that neutralize HIV RT was detected in about 20% of seropositive samples under these assay conditions.

Stabilization assay of human sera or plasma for RT Then we examined whether seropositive or seronegative sera and plasma inhibited heat inactivation of HIV RT. We used the stabilizing index described above to express the effects of sera or plasma on heat inactivation of RT numerically. The stabilizing indices of the sera or plasma from most AIDS patients, hemophiliacs and homosexuals that were positive for HIV antibody were more than

Table I. Effects of Human Sera on Heat Inactivation of RTs

Clinical status	Stabilizing index (%)				
	HTLV-III$_B$	HTLV-1	BLV	MoMuLV	Purified HTLV-III$_B$ RT
ARC	23.7	0.6	0.7	0.3	41.2
AIDS	60.0	0.5	0.6	0.2	60.3
AIDS	0.9	0.0	0.6	0.4	11.2
ATL	0.9	0.3	0.5	0.3	4.1
Normal	0.6	1.4	0.5	0.3	7.8

Stabilization assays of the four retroviruses were carried out as described in "Materials and Methods." Results of five different subjects are shown. The RT activities of HTLV-III$_B$, HTLV-1, BLV, MoMuLV and the purified HTLV-III$_B$ enzyme after incubation with normal sera at 37°C for 30 min were about 7×10^4, 4×10^3, 1.4×10^5, 6×10^5 and 2×10^4 cpm, respectively.

1%, whereas those of normal humans and patients with ATL were less than 1% (Fig. 2). Twenty out of 23 (87%) seropositive samples showed higher stabilizing indices than those of seronegative subjects. The stabilizing index of one serum from an AIDS patient was 60%, whereas his serum neutralized HIV RT most weakly among seropositive subjects. Sera or plasma with high stabilizing activities, however, did not always show either high neutralizing activities against HIV RT, or high antibody titers against HIV proteins determined by IF using acetone-fixed MOLT-4/HTLV-III$_B$ cell smears (data not shown). It remains to be determined whether there is any association between the stabilizing indices of human sera and clinical manifestation of disease.

Effects of human sera on RTs of other retroviruses We examined whether RTs of other retroviruses were also stabilized by HIV antibody-positive sera. Results for some representative sera are shown (Table I). HIV antibody-positive sera stabilized the RT of HTLV-III$_B$, but not the RTs of HTLV-1, BLV or MoMuLV, which were inactivated in the presence of all the human sera or plasma samples so far tested.

The stabilizing index of each serum sample was similar when HIV[GUN-1], HTLV-III$_B$ or HIV[SF-2] (ARV-2) was used as a source of RT (data not shown). Thus, human sera recognized RTs of different HIV isolates, although the restriction enzyme cleavage patterns of these viruses differ markedly.[2, 3, 20]

Next we examined whether human sera containing HTLV-1 antibody affected the stability of HTLV-1 RT in the stabilization assay. Sera from seven ATL patients and two healthy HTLV-1 antibody-positive subjects neither markedly reduced the RT of HTLV-1 at 37°C, nor stabilized the RT at 56°C (data not shown).

Effects of human sera on purified RT We examined whether human sera recognized RT itself or some *gag* protein associated with the RT. RT was purified from the culture fluids of MOLT-4/HTLV-III$_B$ and U-937/HIV-[GUN-1] cells by ultracentrifugation and successive column chromatographies on DEAE-cellulose and phosphocellulose as described.[23] The purified RT was incubated with HIV antibody-positive sera or normal human sera and examined by using the stabilization assay as described above (Table I). HIV antibody-positive sera stabilized the RT of HTLV-III$_B$. Similar results were obtained in experiments using purified RT of HIV[GUN-1] (data not shown).

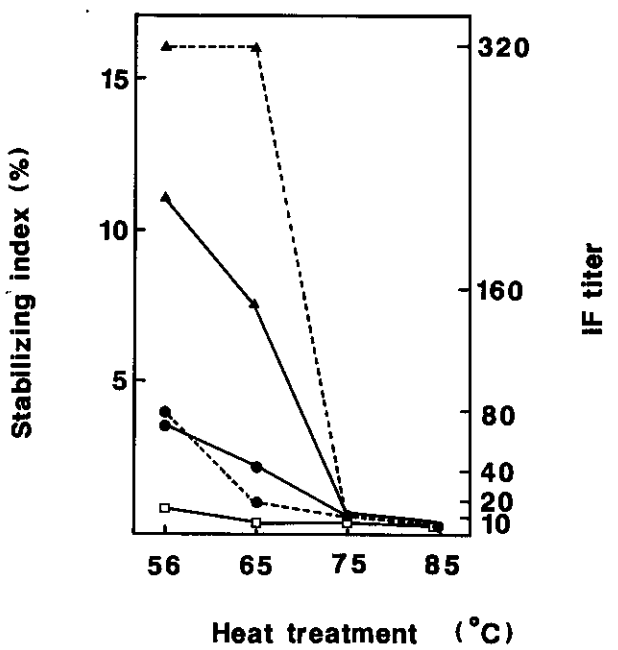

Fig. 3. Effects of heat treatment on the stabilizing activities of human sera. Sera from two ARC patients (● ▲) and a normal subject (□) were heated for 30 min at the indicated temperature (°C) and then their titers were determined by the stabilization assay (solid lines) and IF (broken lines). Sera from the same subjects were absorbed with protein A (Pansorbin cells) and their titers were determined by stabilization assay and IF. IF titers are shown by broken lines.

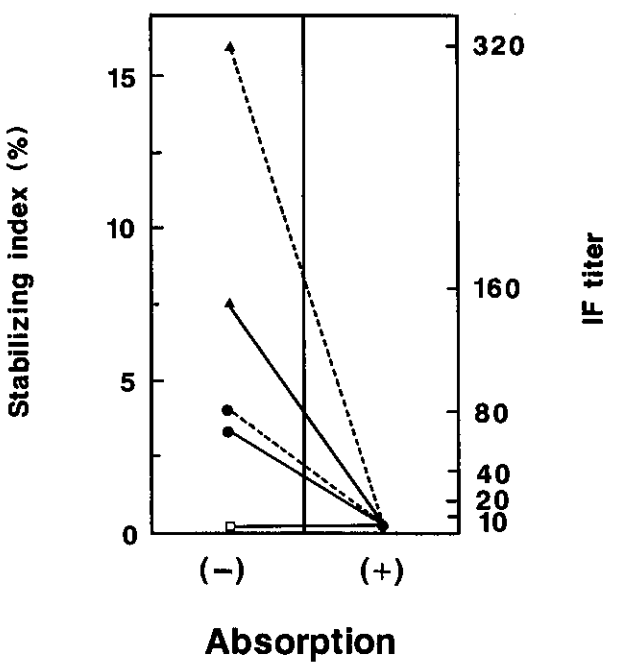

Fig. 4. Effects of protein A absorption on the stabilizing activities of human sera. Sera from the same subjects analyzed in Fig. 3 were absorbed with protein A (Pansorbin cells) and their titers were determined by stabilization assay and IF. Results are shown as in Fig. 3.

Effects of various treatments of sera on the stabilizing indices We examined what factor(s) in human sera or plasma was responsible for stabilization of HIV RT. The stabilizing activities of HIV antibody-positive sera were lost on heating the sera at 75°C or 85°C for 30 min (Fig. 3). Their antibody activities against HIV, detected by IF, were also lost on heat treatment at 75°C and 85°C. The stabilizing activities were also removed from the sera by absorption of the sera with Pansorbin cells (protein A) (Fig. 4), indicating the involvement of IgG in stabilization of HIV RT. In both experiments, there was a good correlation between the stabilizing indices of the treated sera and their IF titers: these two activities of the sera were lost concomitantly during these treatments. These findings suggested that antibody, especially IgG, was necessary for stabilization of HIV RT.

Stabilizing indices of purified IgG IgG fractions were purified with Affigel-Protein A (Bio-Rad) from the sera of two seropositive subjects (ARC and AC), two normal humans and an ATL patient. HIV-seropositive sera that did not markedly inhibit or neutralize HIV RT were selected as shown in Table II. The antibody titers of purified IgG fractions were determined by IF and their effects on stabilization and neutralization of HIV RT were examined (Table II). The IgG fractions purified from HIV antibody-positive sera, but not from sera of normal subjects or an ATL patient, had stabilizing activities. The purified IgGs from the HIV antibody-positive sera did not neutralize the RT activities of HIV lysates at all. Rather, RT activities were detected to be higher in the presence of purified IgG than in its absence.

The mouse monoclonal antibody M3364[7] against RT, obtained in ascites form, also stabilized the RT when added at a concentration of 0.5%, its stabilizing index being 2.7. At higher concentrations of up to 10%, its stabilizing indices were similar. This monoclonal antibody is reported to bind to the RT, but not neutralize it.

Table II. Effects of Purified IgG on Stabilization of HIV RT

Clinical status	IF titer		Stabilizing index (%)		Neutralizing index (%)	
	Serum	IgG	Serum	IgG	Serum	IgG
Normal	<10	<10	0.6	0.5	56	−17
AC	80	20	2.2	2.5	6	−33
ARC	320	160	12.5	10.5	20	−21

IgG was purified from sera of three subjects using Affigel-Protein A columns. Human sera and purified IgG were examined by IF and stabilization and neutralization assays. Each index was calculated as described in "Materials and Methods."

DISCUSSION

There are reports that most HIV antibody-positive sera contain antibody against RT: the *pol* gene product of p66/p51 or p64/p53 has mainly been detected by Western blot analyses.[7, 11, 13, 14, 24] Furthermore, IgG purified from HIV antibody-positive sera neutralized RT.[13, 14] Under our assay conditions, where whole sera or plasma obtained and stored under different conditions were used, only 20% of HIV antibody-positive sera appreciably inhibited HIV RT (more than 80% inhibition). This low frequency of detection of neutralizing antibody might be due to use of whole sera or plasma: concentrations of neutralizing IgG in the samples might be low. The use of whole sera or plasma may not be suitable for neutralization assay of RT. All normal samples were sera obtained from laboratory workers. The difference may have slightly affected the results shown in Fig. 2.

We found that RT was not well inactivated by heat treatment in the presence of sera or plasma containing antibodies, especially IgG, against HIV and that heat inactivation of purified RT partially inhibited by some of the seropositive sera or plasma. These findings suggested that the antibodies against RT could stabilize the enzyme to heat, although it remains probable that antibody against viral structural protein such as *gag* slightly affects the stabilization assay. This stabilization assay can be done with a small amount of serum. Antibody sources used in this experiment were sera or plasma, which had been collected in various laboratories and might have been stored under different conditions. Thus experimental and clinical data of each patient could not be directly compared. Stabilizing indices of ATL patients were lower than those of normal subjects (Fig. 2). ATL samples had come from several hospitals and all of them were plasma. All normal samples were sera obtained from laboratory workers. This difference may have affected the results shown in Fig. 2. Further studies with a larger number of samples of sera from seropositive or seronegative cases and stored under similar conditions are needed to determine how the stabilizing index of each patient fluctuates during the clinical course of the disease. The use of not only neutralization assays of HIV RT, but also stabilization assays may be helpful to estimate the clinical courses of some HIV-infected subjects. Our preliminary assays indicated a significant correlation between CD4 cell numbers and stabilizing or neutralizing activities of HIV-seropositive subjects.

Our findings suggested that inactivation of RTs of retroviruses on heat treatment at 56°C may be due to the presence of heat-labile structures in the enzymes. Some antibody prevents breakdown of these structures during heat treatment. Results of Western blot analyses show that most HIV-seropositive subjects carry antibodies

against RT.[7] We could confirm this finding using more than 100 sera obtained from one hospital. We further noticed that all sera scored positive by either neutralization assay or stabilization assay gave discrete bands upon Western blotting and that some sera gave negative results in both assays, although they were clearly positive by Western blotting (unpublished data). There were also often discrepancies between neutralizing activities and stabilizing activities of samples as described in the text. Therefore, some antibodies may neutralize HIV RT and others may stabilize HIV RT upon heat-inactivation. There may be three types of antibodies against RT in humans that recognize different regions of RT: the first and second types may recognize regions involved in neutralization and stabilization, respectively, of the enzyme, whereas the third and major type may bind to RT, but may not neutralize or stabilize it.

Stabilizing antibodies may be useful for structural analyses of HIV RT. The assay described here could be applicable for detecting antibodies against heat-labile proteins.

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