Antibodies against reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) have been detected in seropositive subjects by immunoprecipitation, Western immunoblotting, and neutralization assay. Recently, we noticed that the antibodies against RT stabilized RT upon heat inactivation, and we have developed a stabilization assay of RT antibody. Briefly, the RT of HIV-1 is completely inactivated by incubation at 56°C for 20 min, but this inactivation is inhibited in the presence of a specific antibody directed against this molecule. We examined the specificity and clinical significance of this stabilization assay. HIV-1 antibody-positive sera stabilized HIV-1 RT but not HIV-2 RT, whereas half of these sera cross-neutralized HIV-2 RT. Antibody titers against RT determined by the neutralization assay and the stabilization assay were compared with clinical characteristics. Antibodies against HIV-1 RT were much more frequently detected by the stabilization assay than by the neutralization assay. Statistically significant associations were found between stabilizing antibody titer and CD4 cell number in peripheral blood of patients and also between antibody titer and CD4⁺/CD8⁺ ratios. These results indicate that our new stabilization assay to detect specific antibodies against RT of HIV-1 is useful as a clinical marker of infection and progress of the disease.

Characteristics and Clinical Significance of a Stabilization Assay To Detect Specific Antibodies to Reverse Transcriptase of Human Immunodeficiency Virus

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Antibodies against reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) have been detected in seropositive subjects by immunoprecipitation, Western immunoblotting, and neutralization assay. Recently, we noticed that the antibodies against RT stabilized RT upon heat inactivation, and we have developed a stabilization assay of RT antibody. Briefly, the RT of HIV-1 is completely inactivated by incubation at 56°C for 20 min, but this inactivation is inhibited in the presence of a specific antibody directed against this molecule. We examined the specificity and clinical significance of this stabilization assay. HIV-1 antibody-positive sera stabilized HIV-1 RT but not HIV-2 RT, whereas half of these sera cross-neutralized HIV-2 RT. Antibody titers against RT determined by the neutralization assay and the stabilization assay were compared with clinical characteristics. Antibodies against HIV-1 RT were much more frequently detected by the stabilization assay than by the neutralization assay. Statistically significant associations were found between stabilizing antibody titer and CD4⁺ cell number in peripheral blood of patients and also between antibody titer and CD4⁺/CD8⁺ ratios. These results indicate that our new stabilization assay to detect specific antibodies against RT of HIV-1 is useful as a clinical marker of infection and progress of the disease.

Materials and Methods

Virus lysates. HIV-1 and HIV-2 were prepared from MOLT-4 (clone 8) HIV-1HTLV-IIIb or (clone 8) HIV-1LA (11, 17) and from MOLT-4 (clone 8) HIV-2ClH-I (10) cells, respectively, which had been maintained in RPMI 1640 medium containing 10% fetal calf serum. The culture medium was centrifuged at 2,500 × g for 15 min at 0°C, and culture supernatants (75 ml) were mixed with 36 ml of 30% polyethylene glycol and 3 ml of 4 M NaCl. The mixtures were incubated on ice for 2 h and centrifuged at 2,500 × g for 30 min at 0°C. The precipitates were suspended in a mixture of 2 ml of suspension buffer (25 mM Tris-HCl [pH 7.5], 50 mM KCI, 50% glycerol, 0.025% Triton X-100, 5 mM dithiothreitol) and 1 ml of lysis buffer (1.5 M KCl, 0.05% Triton X-100). Virus lysates were stored at -80°C.

Human sera. Seropositive samples from 117 adult Caucasians were examined (111 males and 6 females). Ten males had been infected by transfusion of HIV-1-infected factor VIII, and five males had been infected with HIV-1 through the sharing of intravenous needles. The remaining males were infected through homosexuality. All of the females had been infected by transfusion of HIV-1-infected blood prior to the introduction of antibody screening. The median age of the population studied was 36.2 years. Sera used for the studies had been obtained as part of another study looking at cellular markers of HIV disease progression as described previously (1). The sera were collected during the period 1987 to 1988 and had been stored in a freezer at -20°C. In accordance with the therapeutic recommendations in place at that time, only patients with a CD4 count of less than 200 cells and AIDS received antiretroviral therapy (zidovudine). Seropositive patients infected with HIV-1 were classified by Centers for Disease Control (CDC) criteria: AC group II, and ARC group III, and group IV (subgroups A, B, and C). AC group II comprised 43 patients who were asymptomatic and had CD4/CD8 ratios of 1.27 ± 0.75 (mean ± standard deviation [SD]). ARC group III included 29 symptomatic patients with CD4/CD8 ratios of 0.90 ± 0.51. Group IV, i.e., CDC IV subgroup A and AIDS (subgroups B and C), comprised 41 patients with CD4/CD8 ratios of 0.49 ± 0.46. Five African serum samples were positive for antibodies against HIV-2; they were also positive for anti-HIV-1 antibodies by the particle agglutination test (Serodia HIV; Fujirebio, Inc., Tokyo, Japan); their particle agglutination titers were 256 (four samples) and 16,384 (one sample). These sera were determined to be HIV-2 positive by the PEPTI-LAV 1.2 identification assay (Diagnostics Pasteur, Paris, France). All seropositive samples were obtained from 16 Japanese laboratory workers. All sera were thawed, divided into small aliquots, and stored at -70°C until use. Samples were heated at 56°C for 30 min before use.

RT assay. The virus lysates prepared as described above were subjected to the RT assay, which was performed as described elsewhere (9, 16). The RT reaction
RESULTS

Heat inactivation of RT. The RT activities in the virus lysates (20 μl) prepared from culture media of MOLT-4 cells infected with HIV-1HTLV-IIIB and HIV-2GH-1 without heat treatment were about $2 \times 10^5$ cpm. To examine the time course of heat inactivation of RT, the virus lysates were incubated for 10, 20, 40, 60, or 100 min at 56°C. Each sample was quickly cooled on ice after heat treatment, and residual RT activities were then measured.

Time course studies showed that the RT activity of HIVHTLV-IIIB was almost completely lost after heat treatment at 56°C for 20 min (Fig. 1A). However, the RT activity of HIV-2GH-1 was inactivated only up to 95% after treatment at 56°C for 20 min and completely inactivated after treatment for 40 min (Fig. 1A).

Effects of human sera on HIV RT. We showed that heat inactivation of HIV-1 RT was inhibited by sera positive for antibodies against HIV-1 as reported previously (15). Further, the effects of human serum concentrations on heat inactivation of RT were examined (Fig. 1B). HIV-1 and HIV-2 lysates were heated at 56°C for 20 and 40 min, respectively, in the presence of HIV antibody-positive human sera, and the residual RT activities were measured. In the presence of four independent HIV-1 antibody-positive sera at a concentration of 10%, 20 to 100% of HIV-1 RT activities were not inactivated after treatment at 56°C for 20 min (Fig. 1B). Most sera inhibited heat inactivation of HIV-1 RT even at a concentration of 0.6%. Thus, HIV-1 antibody-positive sera stabilized HIV-1 RT upon heat inactivation. However, 16 seronegative serum samples did not inhibit RT inactivation. Similarly, HIV-2 antibody-positive human sera specifically stabilized heat inactivation of HIV-2GH-1 RT (see Fig. 3A).

Stabilization and neutralization assays of human sera for RT. HIV-1 lysates (20 μl) containing RT were incubated at 37 or 56°C in the presence of 2 μl or 5% of human sera or sera from patients with AC, ARC, or AIDS (Fig. 2). The RT activities remaining after heat treatment were measured. The stabilizing activities of the sera from the majority (58%) of CDC group II to group IV patients were more than 1.2% (the mean + 3SD of values of seronegative sera) (see Table 1), whereas those from seronegative subjects were less than 1.2%. Sera from HIV-2 antibody-positive subjects did not stabilize HIVHTLV-IIIB RT (Fig. 2A). HIV-1 antibody-positive subjects were classified into two groups according to their neutralizing activities against HIVHTLV-IIIB RT: one group with values higher than 39% (mean + 3SD of values of seronegative human sera), and one group judged as having no neutralizing activities (Fig. 2B). Neutralizing activities of sera from many HIV-1-infected subjects, especially those in CDC group IV, were even lower than those of sera from normal subjects (Fig. 2B). The reason for this is not clear. HIV-2 antibody-positive sera neutralized HIV-1 RT (Fig. 2B), suggesting cross-neutralization between HIV-1 and HIV-2, while results of stabilization assay showed that four of five antibody-positive sera were nega-
tive for antibody against HIV-1 RT (Fig. 2A). Thus, the stabilization assay was more specific than the neutralization assay.

Next, we examined whether HIV-1- or HIV-2-seropositive sera inhibited heat inactivation of HIV-2\textsubscript{GH-1} (Fig. 3). HIV-1 antibody-positive sera did not stabilize the RT of HIV-2\textsubscript{GH-1}, which was also inactivated in the presence of seronegative human sera. In addition, half of HIV-2 antibody-positive sera stabilized HIV-2\textsubscript{GH-1} RT, and high levels of neutralizing activities were also detected in all HIV-2 antibody-positive sera (Fig. 3). In contrast, about half of the HIV-1 antibody-positive sera were judged to be positive for neutralization of HIV-2\textsubscript{GH-1} RT by this criterion, suggesting cross-neutralization of HIV-1 RT. Neutralizing activities of sera from patients in CDC groups II, III, and IV against HIV-2 RT were significantly higher than those of sera from normal subjects (\(P < 0.001\) to 0.01). Again, the stabilization assay was more specific than the neutralization assay in measuring the reactivity of HIV-1 antibody-positive sera against HIV-2 RT.

Detection of antibodies against HIV-1 RT. The results of assays of stabilizing and neutralizing activities against HIV\textsubscript{HTLV-IIIB} RT are summarized in Table 1. Of 117 seropositive samples, 66 (56\%) showed higher stabilizing activities than the mean + 3SD of seronegative human sera. Statistical analyses (Student’s \(t\) test) were done for each of the HIV-1-seropositive groups, as shown in Table 1. That is, statistically significant differences were found between CDC groups II and
IV ($P < 0.05$) and CDC groups III and IV ($P < 0.01$) in the stabilization assay and between CDC groups II and IV ($P < 0.01$) and CDC groups III and IV ($P < 0.01$) in the neutralization assay. Rates of sera showing neutralizing activities at levels higher than the mean + 3SD of seronegative human sera were 25, 42, 45, and 12% among patients in CDC groups I, II, III, and IV, respectively. HIV-1-antibody-positive sera with stabilizing activities at levels higher than the mean + 3SD of

**TABLE 1. Detection of antibodies against HIV-1 RT by neutralization and stabilization assays**

<table>
<thead>
<tr>
<th>Assay</th>
<th>HIV seronegative</th>
<th>HIV-1 seropositive</th>
<th>HIV-2 seropositive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples with activity &gt; mean + 3SD/no. of samples tested (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralization</td>
<td>0/16 (0%)</td>
<td>1/4 (25%)</td>
<td>18/43* (42%)</td>
</tr>
<tr>
<td>Stabilization</td>
<td>0/16 (0%)</td>
<td>1/4 (25%)</td>
<td>24/43* (56%)</td>
</tr>
</tbody>
</table>

*Significantly different from CDC group IV ($P < 0.01$).

*Significantly different from CDC group IV ($P < 0.05$).
TABLE 2. Correlation coefficients between stabilization or neutralization assay and clinical characteristics of patients

<table>
<thead>
<tr>
<th>Correlation coefficient for:</th>
<th>Neutralization assay</th>
<th>Stabilization assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC stage</td>
<td>-0.225</td>
<td>-0.122</td>
</tr>
<tr>
<td>CD4⁺ count</td>
<td>0.159</td>
<td>0.230</td>
</tr>
<tr>
<td>CD8⁺ count</td>
<td>-0.129</td>
<td>-0.104</td>
</tr>
<tr>
<td>CD4⁺/CD8⁺ ratio</td>
<td>0.126</td>
<td>-0.195</td>
</tr>
<tr>
<td>Neutralization</td>
<td>1.000</td>
<td>0.226</td>
</tr>
<tr>
<td>Stabilization</td>
<td>0.226</td>
<td>1.000</td>
</tr>
</tbody>
</table>

* Pearson's correlation coefficient (P < 0.05).

Correlation coefficients between stabilization and neutralization assays and clinical characteristics of patients. We examined the relationships between the stabilizing or neutralizing activities of patient sera and their clinical characteristics (Table 2). The stabilizing activities showed statistically significant correlations (P < 0.05) with CD4⁺ cell numbers, CD4⁺/CD8⁺ ratios, and the neutralizing activities. The neutralizing activities showed significant correlations (P < 0.05) with CDC stages and stabilizing activities. Thus, antibody titers against HIV-1 RT determined by the stabilization assay or the neutralizing assay were correlated with different clinical parameters of the patients.

Relationship between stabilization assay and Western blotting. We then compared the detection of p66 and p51 Pol proteins by Western blotting with results of stabilization assays. Sera from patients with stabilizing activities over 1.2% (mean + 3SD of seronegative human sera) showed both p66 and p51 bands, while those showing low stabilizing activities (<1.2%) mostly showed neither band or only the p66 band (Fig. 4).

We further examined 44 samples positive for HIV-1 RT antibody and 1 sample negative for this antibody, which had been judged by Western blotting, with regard to the relationship with stabilizing and neutralizing activities (Table 3). Among the 44 samples, only 17 serum samples (39%) showed high neutralizing activities while 32 (73%) gave high stabilizing activities.

These results indicated that there is a high degree of correlation between the reactivity of the sera with p66 and p51 bands and their stabilizing activities. However, the neutralizing activities did not correlate well with the patterns obtained on Western blotting.

DISCUSSION

The RT activity of a lysate of HIV-1 was almost completely inactivated by incubation at 56°C for 20 to 30 min. We previously showed that the heat inactivation of RT in the virus lysate or purified RT was partially inhibited in the presence of some human serum or plasma samples containing antibodies against HIV. The immunoglobulin G purified from seropositive sera was responsible for stabilization of RT upon heat inactivation (15), and the immunoglobulin G purified from HIV antibody-
positive sera was also reported to neutralize HIV-1 RT activity (13, 20). In this paper, we report the specificity and clinical significance of this stabilization assay. We used whole sera or plasma for the assays of their stabilizing and neutralizing activities of crude RT preparations. These assays detected antibodies against RT in a small volume of serum. Only 56% of 117 HIV antibody-positive samples showed higher stabilizing activities than the mean + 3 SD of normal human sera. Particularly, the majority of samples from CDC group III patients showed higher stabilizing activities (CDC group III, 79%). Furthermore, some sera from patients at advanced CDC stages did not contain neutralizing antibodies as reported previously (13, 20), while stabilizing antibody titers persisted in these sera. To determine how the stabilizing index of each patient fluctuates during the clinical course of the disease, further studies with a large number of samples from sera from seropositive and seronegative individuals will be required.

Antibodies against the RT of HIV-1 were much more frequently detected by the stabilization assay than by the neutralization assay. Values obtained by the stabilization assay were more clear-cut than those obtained by the neutralization assay (Fig. 2 and 3) when whole sera were used. The neutralization assay may have given clearer results if purified immunoglobulin G had been used, as reported by Laurence et al. or Sano et al. (13, 20). The amounts of sera we could obtain from different individuals were often very limited. The stabilization assay is suitable for analyses of samples with limited volumes and also is less laborious than the neutralization assay, because whole sera can be used. When these values were compared with the clinical data for the patients, the neutralizing activities showed a correlation with the CDC groups while the stabilizing activities showed significant correlations with CD4 + cell numbers and CD4+/CD8+ ratios. The use of not only the stabilization assay of HIV RT but also of the stabilization assay may be helpful in estimating the clinical courses of some HIV-infected subjects.

Western blotting analyses have often been used to detect antibody against Pol proteins (p66/p51 or p64/p53) (6), and the intensities of p66 and p51 protein bands correlated with the stabilizing activities in sera (Fig. 4). We further noticed that all sera that scored positive by either the neutralization assay or stabilization assay gave discrete bands on Western blotting and that not all sera gave negative results in both assays although they were clearly positive by Western blotting. There were often discrepancies between neutralizing activities and stabilizing activities of samples as described above. Therefore, RT has multiple antigenic sites or epitopes. The epitopes recognized by the stabilizing antibody may be different from those recognized by the neutralizing antibody. Some antibodies may neutralize HIV RT, and others may stabilize the enzyme against heat inactivation. There may be at least three types of antibodies 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 type may bind to RT but with neither neutralizing nor stabilizing activity.

Our findings suggested that inactivation of retrovirus RT on heat treatment at 56°C is due to the presence of heat-labile structures in the enzymes. Some antibodies prevent breakdown of these structures during heat treatment. We also examined the cross-reactivity of the stabilizing or neutralizing antibodies by using the RT from HIV-1HTLV-IIIB and HIV-2GH-1 and found specific reactions of positive sera with the corresponding viruses. These results indicated that the RT of HIV-1 and HIV-2 can be distinguished by the stabilization assay with sera from patients infected with HIV-1 or HIV-2.

The assay described here could be applicable for detecting antibodies against a variety of heat-labile proteins. Stabilizing antibodies may also be useful in analyzing the structure of HIV RT. The stabilizing antibody is a new assay to detect specific antibodies against RTs of HIV-1 and HIV-2.

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