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Measurement of Levels of Human Immunodeficiency Virus Type 1 Reverse Transcriptase (RT) and RT Activity-Blocking Antibody in Human Serum by a New Standardized Colorimetric Assay

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Standardization and calibration of a new colorimetric assay for detection of reverse transcriptase (RT) was carried out for optimal detection of RT activity-blocking antibody (RTb-Ab) in serum. A total of 99 of 100 Swedish and 54 of 54 African human immunodeficiency virus type 1 (HIV-1) antibody-positive individuals had RTb-Ab. The one RTb-Ab-negative HIV-1 serum sample from a Swedish individual was obtained early during seroconversion. Five of 615 HIV-1-negative sera from tumor patients, pregnant women, patients undergoing routine viral diagnostics, and blood donors gave false-positive results. In addition, 3 of 126 HIV-1-negative African serum samples and 2 of 91 serum samples selected because of false reactivity in other commercially available HIV antibody assays were positive for RTb-Ab. RT activity and RTb-Ab were measured in sera from newly HIV-1-infected individuals during seroconversion. Peak RT activity was usually detected between days 8 and 13 after the onset of symptoms of primary infection. In addition, HIV-1 RTb-Ab was detected in the same recently infected individuals in most cases within 1 month and in some cases as early as 10 to 12 days after the onset of symptoms. A cross-reactivity study involving HIV-1 and HIV-2 RTb-Abs and their homologous RT showed HIV-1 RTb-Ab to be highly type specific. None of 10 serum samples from HIV-1-infected individuals showed cross-reacting RTb-Ab toward HIV-2 RT, whereas 4 of 10 serum samples from HIV-2-infected patients showed cross-reactivity toward HIV-1 RT; however, the cross-reactivity toward HIV-1 RT was 3,000 times lower than that toward its homologous RT. Future uses for the assay with reference to the recent World Health Organization proposal for other methods instead of Western blotting (immunoblotting) for confirming HIV-1 infection and for methods for the diagnosis of infection as follow-up in vaccine trials are also discussed.

During the acute phase of human immunodeficiency virus type 1 (HIV-1) infection a rapid replication of virus occurs, leading to a viremia. During this viremic phase HIV-1 antigen is present in high amounts in the infected individual's bloodstream. Only after the induction of the specific immune response, namely, cytotoxic T lymphocytes and HIV-1-specific antibodies, does the viremia decline. The time period from initial infection with HIV-1 and the first appearance of antibodies directed against viral membrane proteins gp41, gp120, and gp160 is generally less than 3 months (16). Traditionally, enzyme immunoassays are the serological tests most commonly used for the diagnosis of HIV-1 infection. Such tests are often simple to perform and their results are easy to interpret; however, even though they are highly accurate, some sera can give false-positive results. Therefore, the use of supplementary tests is necessary to confirm or reject positive results, with the most common of these being the Western blot (immunoblot) assay (WB).

HIV encodes for an RNA-dependent DNA polymerase, the reverse transcriptase (RT), which is essential for the replication of HIV (6). RT transcribes the viral RNA into viral DNA prior to its insertion into the host cell genome and the establishment of proviral DNA. This proviral DNA contains the genetic information required for the production of virus-specific proteins, including the RT. The detection of RT activity is widely used to monitor HIV replication in cell culture and has also recently been measured in serum during the acute viremic phase of simian immunodeficiency virus (SIV) infection in macaques (8). Although the RT is carried within the nucleocapsid core of HIV-1, high levels of antibody (Ab) directed against RT have been shown in infected individuals (1, 5, 9), even as early as the time of appearance of gp41 Abs (21). Falling RT-Ab titers have also been shown to be a predictive marker for disease progression (1, 3, 15, 21, 22, 24).

Measurement of RT-Ab can be carried out in two ways: first, by the use of inactive RT proteins as antigen in standard enzyme-linked immunosorbent assays (ELISAs) (19) or, second, by the use of catalytically competent enzyme. In the latter case, RT-Ab which blocks the catalytic capacity of RT (RTb-Ab) is measured. These RTb-Abs have been determined in small sample collections by several groups (1, 3, 15, 24, 26). Initially, RTb-Ab was determined by rather laborious, insensitive techniques that required large amounts of RT for activity determination. Large amounts of RT subsequently resulted in the requirement for a large quantity of RTb-Ab to inhibit the activity. Since high concentrations of serum tend to interfere with RT assays, these earlier studies required the purification of the immunoglobulin G fraction of each serum sample tested (3). Recent work has produced a new generation of highly sensitive and specific methods for the evaluation of RTb-Ab in

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minute, unpurified serum samples (21, 22, 26). The ability of an assay to detect RTb-Ab inherently depends on the amount of RT used. The less RT included, the smaller the amount of RTb-Ab required to block the enzyme and the higher detection sensitivity of the assay. The current highly sensitive RT assay based on carrier-bound template coupled to the bottom of the wells and 5-bromodeoxyuridine 5′-triphosphate (BrdUTP) as the deoxynucleoside triphosphate has been adapted to a conventional 96-well microtiter plate format. The nonradioactive detection step involves the binding of alkaline phosphatase-conjugated anti-BrdU antibody instead of the more traditional tritiated or iodinated triphosphate used earlier, followed by a colorimetric step (7).

This study focuses on the standardization of the current RT assay for the optimal detection of RTb-Ab in serum samples. Specificity and sensitivity studies were carried out for the detection of RTb-Ab in defined panels of sera, including sera from newly HIV-1-infected individuals directly after the onset of symptoms of primary infection. In addition, results of a cross-reactivity study performed to determine the extent of cross-reactive RTb-Abs from the sera of patients with different retroviral infections, namely HIV-1 and HIV-2, are also presented.

MATERIALS AND METHODS

Chemicals, enzyme source, and clinical material. The assay used in the study was supplied as a complete kit (Retrosys) by Innovagen AB, Lund, Sweden. Recombinant HIV-1 RT was supplied as part of the Retrosys kit. Cell culture-derived HIV-2 RT was supplied by Medivir AB, Stockholm, Sweden. The HIV-2 strain (strain SBL 6669) used in the study was propagated in the T-cell line MT-4. The cells were maintained in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. When more than 25% of the cells exhibited signs of cytopathic effect, the supernatants were collected, tested for RT activity, and frozen in aliquots as virus stocks. Consecutive patient and blood donor sera from Tanzania were obtained through a research collaboration between the Swedish Institute for Infectious Disease Control (SIIDC), Stockholm, and Muhimbili Medical Center, Dar es Salaam, Tanzania. A total of 54 of the serum samples were confirmed to be HIV-1 antibody positive by WB and 126 were HIV antibody negative. HIV-2 antibody-positive samples ($n = 10$) were obtained through a collaboration with the Public Health Laboratory, Bissau, Guinea Bissau. A total of 100 HIV-1 antibody-positive samples were collected from homo- and bisexual men at various stages of HIV-1 infection, from seroconversion to AIDS. Consecutive samples from nine individuals recently infected with HIV-1 collected between days 4 and 407 after the onset of symptoms of primary infection (Table 1) were collected through a collaboration with the Clinic for Infectious Diseases, Huddinge Hospital, Stockholm, Sweden. All serum samples used for the assay evaluation were coded and tested blind. The code was not broken until all samples were analyzed and the results were summarized. The serum panels have also been used for the evaluation of commercially available HIV antibody assays (evaluation of nine commercially available combined anti-HIV-1 and anti-HIV-2 ELISAs, SIIDC, 1995). The reference assays used were the Abbott p24 antigen enzyme immunoassay and IMx HIV-1/HIV-2 3rd Gen (Abbott Diagnostics Division, Delkenheim, Germany), Enzygnost HIV-1/HIV-2 Plus (Behringwerke AG, Marburg, Germany), and an HIV-1 WB (Diagnostic Biotechnology, version 2.2; Genelabs Diagnostic, Singapore). The World Health Organization (WHO) criteria for WB positivity were followed. These criteria require reactivity with at least two *env* bands (28). Sera from blood donors ($n = 100$), sera from women in the first trimester of pregnancy screened for rubella virus immunity ($n = 200$), and sera sent for routine viral diagnostics apart from HIV ($n = 200$) were obtained from the Virus Diagnostics Laboratory at the Department of Clinical Virology, University of Uppsala, Uppsala, Sweden. Sera from cancer patients ($n = 115$), mainly with lymphomas and leukemias, referred for thymidine kinase measurement were also included (sera giving low, medium, and high thymidine kinase activity values were used). Defined problematic sera ($n = 91$) selected for their ability to give false-positive results in other commercially available HIV Ab assays were also supplied.

RT activity assay. The RT assay (Retrosys) used in this study is described in detail by Ekstrand et al. (7). This study involved the use of the assay in the commercially available kit format. In principal, 50 μl of serially diluted sample (1/5, 1/25, and 1/125) is added to a 96-well microtiter plate, with poly(rA) coupled to the bottom of the wells as the enzyme template and 150 μl of reaction solution consisting of oligo(dT) as the primer and BrdUTP as the deoxynucleoside triphosphate substrate. The final amount of sample in each well corresponds to 10, 2, and 0.4 μl of pure serum. A total of 50 μl of sample dilution buffer is also added to several wells of the plate (minimum of four) containing the reaction solution. These are the background controls. Sample dilution buffer for direct RT activity determination consists of HEPES (10 mM), $MgCl_2 \cdot 6H_2O$ (4 mM), dextran sulfate (50 mg/ml), and Triton X-100 (1%). RT, if present in the sample, catalyzes the polymerization of a new DNA strand along the poly(rA) template consisting of incorporated bromodeoxyuridine monophosphate. Polymerization is allowed to proceed for a desired period of time, after which the plate is washed to remove unused substrate. In our case we used an overnight (18 to 20 h) polymerization step. Product detection is carried out immunologically by adding alkaline phosphatase (AP)-conjugated anti-BrdU Ab to each well for 90 min at 33°C. The plate is then washed, and this is followed by a colorimetric determination of the bound Ab with an AP substrate, *para*-nitrophenyl phosphate. The intensity of the color reaction is read in a standard plate reader (405 nm) at defined times, usually at times of 0.5, 1, 2, 4, and 24 h. The amount of RT present in each sample dilution is then calculated as the concentration (in picograms per milliliter) by using a serially diluted reference enzyme with a known RT concentration on all plates. The concentration in each sample dilution is then used to calculate the mean concentration in the undiluted sample. All serum samples with RT activities corresponding to ≥4 pg/ml and having absorbance signals greater than two times the background value (i.e., if the mean of the background controls at a given reading time is 0.05 absorbance units, a positive RT activity in the samples would have to have a minimum signal of 0.1 absorbance units) are deemed positive for RT activity. RT activity of <4 pg/ml is considered nonspecific interference because background noise of up to 4 pg/ml can be detected by the assay in some non-HIV-infected sera. This interference has been experimentally attributed to some sera which have the capacity to cause the AP-conjugated anti-BrdU Ab (kit tracer Ab) to bind nonspecifically to the wells.

Innovagen AB holds the license for the patent (SE 8804344-3) on the carrier-bound template used in the Retrosys assay.

Inactivation of sera. All work involving noninactivated material was carried out at the safety laboratory, SIIDC. All other work was done at the Research Unit for Replication Enzymology, Department of Medical Genetics, Uppsala University, Uppsala, Sweden. Sera tested for RTb-Ab were first pretreated to render them noninfectious and to destroy potential exogenous and endogenous polymerase activity. A total of 75 μl of sample was added to a tube containing 25 μl of 5% Triton X-100 in distilled water. Each tube was incubated for 30 min at 56°C in a water bath, cooled, and assayed for the presence of RTb-ab.

Determination of RTb-Ab. The measurement of RTb-Ab is based on the blocking of the RT enzyme. The sensitivity of an assay for the detection of RTb-Ab depends on the amount of RT used. Since the Retrosys assay can use a very small amount of RT, it gives a very high detection sensitivity for RTb-Ab. In principle, samples are incubated with a standardized small amount of recombinant HIV-1 RT for 90 min at 33°C. RTb-Ab, if present in the sample, will bind and block the catalytic activity of the RT enzyme. Residual RT activity in the sample-RT mixture is then determined by using an overnight polymerization step and a 2- to 3-h colorimetric reading time in the same manner outlined above. For RTb-Ab endpoint titer determination, the sample (heat inactivated, as outlined above) is diluted by using seven 10-fold dilution steps. The first sample dilution in the series (the original heat-inactivated sample) is termed 1/1, the second dilution is termed 1/10, the third dilution is termed 1/100, and so on until the seventh dilution, which is 1/10,000,000. The composition of the sample dilution buffer used is HEPES (4.99 mM), $MgCl_2 \cdot 6H_2O$ (1.99 mM), newborn calf serum (1%), Triton X-100 (1%), and NaN_3 (1.92 mM). Equal volumes of each sample dilution and RT are incubated as described above, after which 100 μl of the sample-RT mixture is transferred to the poly(rA) plate containing 100 μl of reaction solution [the first dilution of serum (1/1) corresponds to 4.4 μl of pure serum added to the well of the poly(rA) plate]. After polymerization and colorimetric detection and after correction for background signal, the residual RT activity (in percent) of each sample dilution is expressed relative to that of the controls with 100% activity included on all plates. The RTb-Ab endpoint titer of a sample is defined as the dilution of the serum which blocks 50% of the activity of the recombinant RT (rRT) added to the assay. The RTb-Ab titer of a sample is calculated by plotting the residual RT activities for each of the sample dilutions to the $\log_{10}$ of each sample dilution (0 to 7) (see Fig. 6A and C), after which the $\log_{10}$ dilution of the sample corresponding to a 50% reduction in RT activity is extrapolated from the plotted curve. The reciprocal of this dilution is the endpoint titer of the sample.

For direct screening for RTb-Ab, undiluted samples are used (allowing for the inherent 1.25 dilution factor incurred during the serum inactivation process). A total of 10 μl of heat-inactivated serum is first incubated with the RT, after which 100 μl is transferred to the poly(rA) plate as described above. This amount corresponds to 4.4 μl of pure serum added to the well of the poly(rA) plate. Samples which give >50% residual RT activity compared to the mean for the 100% RT activity controls after subtraction of the background signal are deemed to be negative for RTb-Ab. Samples giving ≤50% residual RT activity compared to the mean of the 100% RT activity controls after background subtraction were deemed positive for RTb-Ab. Positive samples are classified as strongly positive (<25%), intermediately positive (26 to 35%), and weakly positive (36 to 50%).

Cross-reactivity study. The HIV-2 RT used was initially standardized for RT activity determination prior to use in the RTb-Ab assay. This was carried out by serially diluting the HIV-2 RT preparation in the assay sample dilution buffer and choosing a dilution which gave an RT activity in the same range as the HIV-1

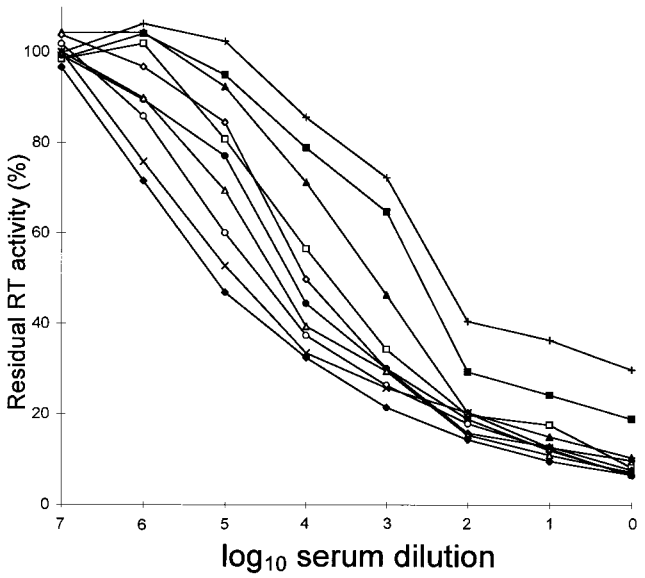

FIG. 1. Dependence on antigen (RT) amount for the sensitive detection of RTb-Ab in serum. The assays were performed by using a 3-h and a standard polymerization step (20 h) with various phosphatase reading times (15 min to overnight). Data from both assays were extrapolated to define titers at high and low RT amounts. +, 300 pg/well; ■, 150 pg/well; ▲, 75 pg/well; □, 35.5 pg/well; ◇, 18.75 pg/well; ●, 9.4 pg/well; △, 4.7 pg/well; ○, 2.34 pg/well; ×, 1.17 pg/well; ◆, 0.58 pg/well.

RT used in the assay. Thereafter, sera from 10 HIV-1-infected and 10 HIV-2-infected patients were evaluated for cross-reactive RTb-Ab by the assay. Standardized amounts of HIV-1 and HIV-2 RTs were preincubated in the presence of equal volumes of serially diluted HIV-1 and HIV-2 Ab-positive sera in the same manner described above for endpoint titer determination. After preincubation, the residual RT activity was measured in the RT-serum mixture. The residual RT activity (in percent) was determined and was used to evaluate the extent of cross-reactivity between HIV-1 and HIV-2 RTb-Abs and their different RTs.

RESULTS

To evaluate the variation in the inhibition of RT activity by serum from HIV-1-infected and non-HIV-1-infected individuals, we used sera from several different sources; HIV-1-infected Swedish and Tanzanian individuals and control sera, some of which were known to cause false-positive results in commercially available HIV Ab assays. However, to do this we first had to determine a standard RT amount to be used in the assay. This was achieved by the experiment described in the next section.

Assay sensitivity as a function of antigen (RT) amount. The capacity of Ab to block an enzyme depends to a large extent on the amount of enzyme. In this respect we incubated various amounts of serially diluted recombinant HIV-1 RT in the presence of serially diluted HIV-1-antibody-positive sera in a chessboard-type experiment. RT concentrations ranging from 300 to 0.58 pg/well were tested. Figure 1 presents the dependence of the antigen (HIV-1 RT) amount for the sensitive detection of RTb-Ab in an HIV-1-antibody positive serum sample. Figure 1 shows that with each sequential dilution of RT a direct increase in RTb-Ab titer was obtained. This was seen with all sera tested. By using the serum sample used for Fig. 1, a RT concentration of 300 pg/well gave an RTb-Ab titer of 200. However, when the RT amount was reduced to 0.58 pg/well, a titer of 140,000 was obtained. Thus, with a 517-fold reduction in RT amount, a 700-fold increase in RTb-Ab titer was achieved. Therefore, to obtain a high assay sensitivity and detection level, a very small RT-antigen amount, corresponding to 1.7 pg of RT/well, was chosen as the standard enzyme amount in all future RTb-Ab assays. This amount, and not the lowest amount of 0.58 pg/well displayed in Fig. 1, was chosen as the amount of antigen, because this concentration gave a signal of 1.6 to 1.8 absorbance units by using the standard polymerization time (20 h) and 2 h of phosphatase reading without any significant reduction in assay sensitivity.

RT inhibitory capacity of control sera. In addition to healthy donor material, samples from other sources which are known to cause complications in standard HIV diagnostic tests were used. Figure 2 presents the distribution of residual RT activity (in percent) for all control sera tested. Of the 615 serum samples screened by the RTb-Ab assay, 5 gave false-positive results. All five serum samples gave residual RT activities of between 25 and 49%. All 100 serum samples from blood donors tested were negative for RTb-Ab. One of 200 serum samples delivered for routine viral diagnostics was positive, while all serum samples from 115 patients with tumor diseases were negative. Four of 200 serum samples from women in the first trimester of pregnancy screened positive.

Twelve serum samples which gave >120% residual RT activity in the RTb-Ab screening assay (Fig. 2) were reanalyzed. The repeated experiments again gave residual RT activity of >120% for all serum samples retested. To rule out the presence of RT in these sera, the screening assay for RTb-Ab was repeated as outlined in Materials and Methods, with the exception that the addition of rRT was omitted. The sera were also tested for the presence of RT activity by the standard RT assay described in Materials and Methods. Neither assay gave RT activity for the 12 serum samples tested. Therefore, we conclude that the sera which gave >120% residual RT activity contained stabilizing and/or stimulating factors which caused the rRT added to the RTb-Ab assay mixture to have increased activity compared to that of the assay's controls with 100% activity. Therefore, the spread of residual RT activities in Fig. 2 can be divided into sera which have inhibitory effects, those which have no effect, and those which have a stimulatory effect on the RT.

HIV-1 RTb-Ab status in sera giving false-positive results in other commercially available HIV Ab assays. The ability of the assay to differentiate between problematic sera and those which are from truly HIV-1-infected individuals was determined. In this respect we screened 91 serum samples which were specifically selected for their ability to give false-positive results in other commercially available HIV Ab assays. All sera were screened for RTb-Ab as described in Materials and Methods. Figure 3 depicts the residual RT activity of the 91 serum samples tested. The sera gave residual RT activities of between 36 and 115%. The majority of sera gave residual RT activities of between 75 and 105%. Of the 91 serum samples tested, 2 screened positive for RTb-Ab. Both of these serum samples were weakly positive in the RTb-Ab assay. Furthermore, both serum samples gave absorbance values two to three times the cutoff in the Abbott IMx and Enzygnost HIV 1/2 Plus assays, but they were subsequently diagnosed as negative by WB and with follow-up samples.

HIV-1 RTb-Ab detection in 100 HIV-1-infected Swedish serum samples. The degree of RT inhibition by HIV-1-infected sera of Swedish origin was determined. Figure 4 presents the residual RT activities for the 100 serum samples. The sera gave residual RT activities of between 0 and 79%. The majority of the sera from this panel gave residual RT activities of 0 to 30%. Of the 100 serum samples, 99 screened positive for RTb-Ab, while 1 serum sample gave a negative result. This serum sample showed one distinct *env* band (gp160) and faint reactivity to

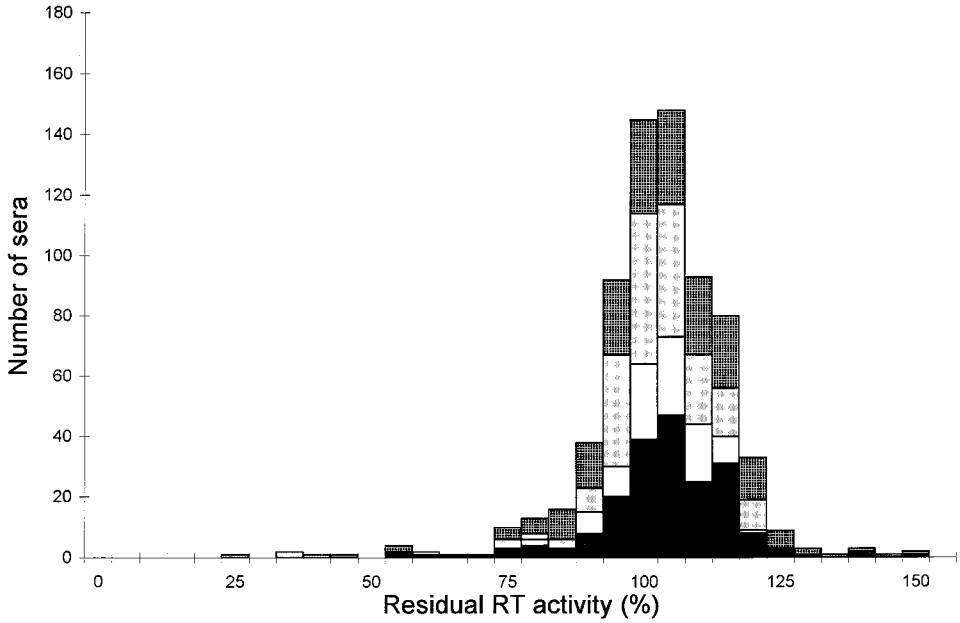

FIG. 2. Distribution of RT-inhibiting capacity of 615 serum samples of Swedish origin. The assay was performed by using standard reaction conditions for screening of sera for RTb-Ab, as outlined in Materials and Methods. □, sera from healthy blood donors; ▨, sera from women in the first trimester of pregnancy; ■, sera delivered for routine viral diagnostics apart from HIV detection; ▨, sera from patients with malignant diseases, mainly leukemia and lymphoma.

p24 and gp120 on WB; thus, it did not attain the WHO criteria for WB positivity (28). Medical records indicated that the serum sample was obtained from an individual during early stages of seroconversion.

HIV-1 RTb-Ab status of 180 serum samples of Tanzanian origin. Because of the high level of immunoglobulin and other unidentified interfering factors in African sera, a high frequency of false-positive results are obtained with some commercially available HIV antibody assays. Because of this, we screened 180 Tanzanian serum samples for RTb-Ab as outlined in Materials and Methods. Figure 5 presents the distribution of residual RT activity for the 180 serum samples. A total of 57 of 180 serum samples screened were positive for RTb-Ab. All sera designated as being positive by conventional HIV Ab assay and WB (n = 54) gave residual RT activities of <35%, the majority of which gave residual RT activities of <10%.

The majority of HIV-1-negative sera gave residual RT activities of between 80 and 100%. Of the 126 serum samples designated as being negative by conventional assay, 123 were negative for RTb-Ab. Of the three false-reactive serum samples, one was WB negative, while the other two serum samples gave indeterminate WB profiles. One of these serum samples showed a faint reactivity with the p51 region, and the other one showed reactivity with the p51 and p55 regions.

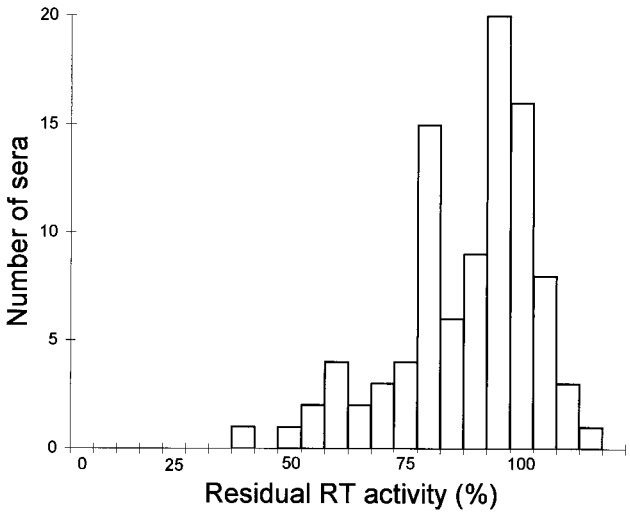

FIG. 3. The RT-inhibiting capacity of 91 serum samples selected for their ability to give false-positive results in standard HIV antibody assays. The RTb-Ab assay was performed by using standard reaction conditions for screening of sera for RTb-Ab as outlined in Materials and Methods.

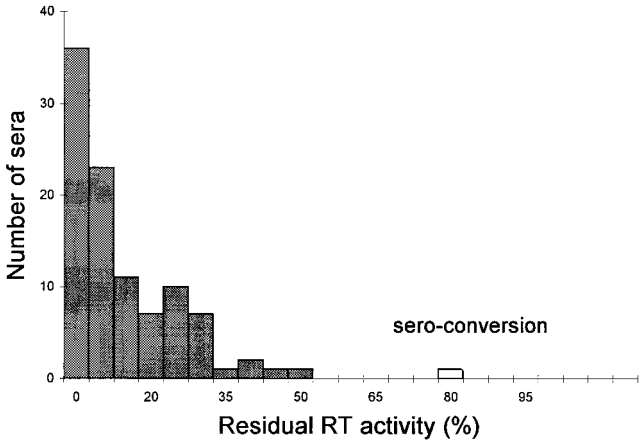

FIG. 4. The RT-inhibiting capacity of 100 HIV-1 antibody-positive sera of Swedish origin. The assay was performed by using standard reaction conditions for screening of sera for RTb-Ab, as outlined in Materials and Methods. ▨, HIV-1-positive sera diagnosed by standard HIV ELISA and WB; □, serum from an individual early during seroconversion.

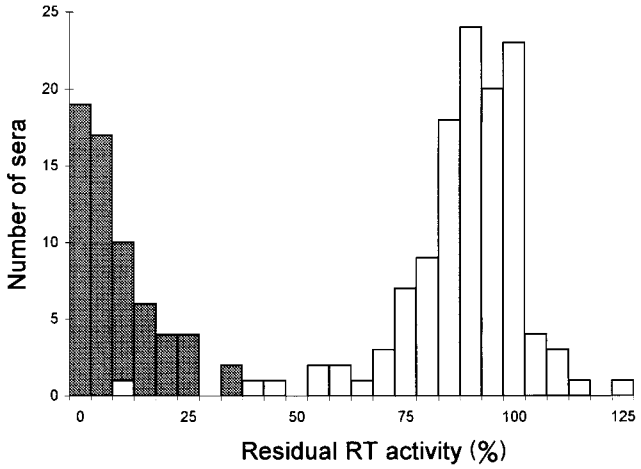

FIG. 5. Distribution of RT-inhibiting capacity of 180 serum samples of Tanzanian origin. The assay was performed by using standard reaction conditions for screening of sera for RTb-Ab as outlined in Materials and Methods. ▨, HIV-1 antibody-positive sera diagnosed by standard HIV ELISA and WB; □, HIV-1 antibody-negative sera diagnosed by standard HIV ELISA and WB.

Detection of HIV-1 RT and RTb-Ab in newly infected individuals directly after the onset of symptoms of primary infection. To evaluate the ability of the assay to detect RTb-Ab in recently infected individuals, sera from nine patients newly infected with HIV-1 collected on a series of days after the detection of symptoms of primary infection were used. RT activity determination and screening for RTb-Ab in each successive patient serum sample was carried out by the RT and RTb-Ab assays as outlined in Materials and Methods. Table 1 summarizes the data compiled on the patients. For the majority of patients, RT activity in the patient serum peaked between days 8 and 13 after the onset of symptoms. This correlates well with the amount of HIV-1 p24 core antigen detected. As the level of RT declined, the patients usually became positive for RTb-Ab; for the majority of patients this was within 1 month after the onset of symptoms. Three of the patients became positive for RTb-Ab at days 53, 58, and 98, respectively. However, for two of these patients samples were not drawn between days 16 and 53 (for patient 4) and between days 22 and 58 (for patient 5), therefore making it impossible to assertain whether positivity was achieved inside the 1-month period. RTb-Ab positivity also showed a good correlation with the appearance of the p51 band on Western blots.

Degree of cross-reactivity between HIV-1 and HIV-2 RTb-Ab and their constituent RTs. To determine the degree of cross-reactive RTb-Ab between HIV-1- and HIV-2-infected individuals, sera from 10 HIV-1-infected individuals and 10 HIV-2-infected individuals were used. All sera were titrated to both HIV-1 and HIV-2 RTs as outlined in Materials and Methods for endpoint titer determination. Figure 6 illustrates the degree of cross-reactivity between HIV-1 and HIV-2 RTb-Ab, and HIV-1 and HIV-2 RTs. HIV-1 RTb-Ab titers ranged from 150 to 110,000 (Fig. 6A). None of the 10 serum samples from HIV-1-infected individuals showed cross-reactivity toward HIV-2 RT (Fig. 6B). The reduction in residual RT activity seen in the highest concentration of sera was not due to cross-reacting RTb-Ab but was caused by serum disturbance. The same disturbance was seen with HIV-negative sera when titrated toward this cell culture-derived HIV-2 RT (data not shown). On the other hand, 4 of 10 serum samples from HIV-2-infected individuals showed cross-reacting RTb-Ab endpoint titers ranging from 5 to 5,300 (Fig. 6D). One of the HIV-2-infected serum samples with low cross-reactivity had very high RTb-Ab endpoint titers toward its homologous RT (i.e., HIV-2 RT) (Fig. 6C). Of the two serum samples with high cross-reactivity, one had very high RTb-Ab endpoint titers toward the homologous RT, whereas the other sample had endpoint titers toward both RTs of equal magnitude. This latter serum sample tested positive for both HIV-1 and HIV-2 by WB, but it was later shown to be HIV-2 antibody positive by a discriminatory peptide-based assay (Pepti-LAV; Diagnostics Pasteur, Marnes la Coquette, France). Although the other three serum samples had cross-reacting RTb-Ab endpoint titers toward HIV-1 RT, the titers were 3,000 times lower than the titers obtained when the homologous RT was used.

Reproducibility data for screening and endpoint titer determination of HIV-1 RTb-Ab. To analyze the reproducibility of the RTb-Ab assay, four control serum samples (HIV negative) and four HIV-1 Ab-positive serum samples were used. Screening for HIV-1 RTb-Ab was carried out by using both groups of sera. The HIV-1 Ab-positive sera were also used for the determination of endpoint titers of HIV-1 RTb-Ab. All sera were analyzed on six separate occasions. Table 2 presents the within-assay mean and standard deviation (SD) and the between-assay mean, SD, and coefficient of variation (CV; in percent) for each serum sample tested. Statistical calculations were carried out as described by Burdon and van Knippenberg (2). The sera used in the screening assays were tested six times in each individual assay run, after which the within-assay mean and SD for each run were calculated. Between-assay variability for the screening assay was determined by using the modified SD formula for replicate samples, $[(\text{SD of the means})^2 - (\text{mean within-assay SD})^2/r)]^{1/2}$, where r is the number of replicates. The SD achieved by using the modified formula was then used to calculate the CV by using the formula CV = (SD/mean) × 100. The sera used in the titer determination assay were tested once in each assay run. Therefore, the standard SD formula for single samples was used to calculate the between-assay SD. The SD achieved was used to calculate the CV by using the formula given above.

DISCUSSION

This report describes a new standardized assay for the detection of HIV-1 RTb-Ab in serum. The assay is based on the detection of antibody which blocks the catalytic activity of the HIV-1 *pol* gene product, RT. This is in contrast to all other commercially available HIV-1 Ab assays in current use, which detect Ab toward the viruses *env* gene (gp160, gp120 and gp41) and *gag* gene (p24) products. The need for supplementary assays based on other antigens and/or test principles prompted us to look at Abs against other HIV-1 gene products and to develop assays for their sensitive detection. Our group had earlier developed assays for the quantitation of RT activity in biological samples (7, 10, 17). It was therefore a simple conversion for the measurement of RTb-Ab. The measurement of Ab catalytically blocking an enzyme activity is inherently dependent on the amount of enzyme used. The RTb-Ab assay uses minute amounts of rRT. Therefore, it allows for the very sensitive detection of RTb-Ab over a wide range of titers.

The assay proved to be highly sensitive for the detection of HIV-1 positivity in both Swedish and Tanzanian patients (99 and 100%, respectively). All but 1 serum sample from a panel of serum samples from 100 Swedish HIV-1-infected individuals screened positive for RTb-Ab. During follow-up of this negative serum sample it was determined that it originated from an HIV-1-infected individual during seroconversion. This serum

TABLE 1. Detection of RT and RTb-Ab in relation to detection by other commercially available antigen and antibody assays in recently HIV-1-infected individuals[a]

Sample no./patient no.	Day post symptoms	RT assay (pg/ml)	Abbott p24 antigen assay (pg/ml)[b]	RTb-Ab assay (%)	Abbott IMx[c]	Enzygnost HIV 1/2 PLUS[c]	WB (band size [bs])
3942/1	5	24	70	74	0.9	ND[d]	160
3943/1	8	27	54	71	1.5	ND	160
3944/1	12	9	112	44	19.0	ND	24, 51, 55, 160
4004/1	15	5	72	16	15.0	ND	24, 51, 55, 64, 160
364/1	78	4	<10	10	14.0	ND	Full profile
1564/2	6	16	104	88	0.7	0.2	NEG[e]
1565/2	7	16	104	72	0.8	0.3	NEG
1566/2	8	22	116	69	1.0	0.6	NEG
1567/2	9	49	90	81	1.5	1.1	160
1568/2	10	28	51	84	2.0	1.7	160
1569/2	11	27	38	78	2.3	1.9	160
1657/2	13	31	19	70	ND	3.4	160
1658/2	14	13	14	84	ND	3.9	(24), 160
1715/2	19	11	19	57	ND	>5	24, 51, 55, 120, 160
1814/2	28	10	<10	49	ND	>5	Full profile
2015/2	42	<4	<10	19	ND	>5	Full profile
1317/2	350	<4	<10	12	ND	ND	ND
535/3	4	<4	185	68	0.5	0.1	NEG
566/3	5	19	356	67	0.5	0.2	NEG
571/3	6	36	197	70	0.8	0.4	NEG
572/3	7	44	110	66	0.9	0.5	NEG
575/3	9	33	65	62	1.4	1.0	NEG
586/3	10	30	ND	49	1.4	1.1	NEG
989/3	407	11	ND	8	ND	ND	Full profile
1228/4	6	22	229	86	0.5	0.1	NEG
1229/4	7	14	ND	89	0.8	0.2	NEG
1230/4	8	13	143	83	1.7	0.3	NEG
1303/4	11	67	1,368	84	3.4	0.9	160
1322/4	12	62	1,267	83	4.7	1.5	160
1325/4	13	68	778	80	7.3	3.5	160
1326/4	14	44	145	87	9.3	>5	(24), 160
1327/4	15	20	109	87	9.9	>5	24, (55), 160
1342/4	16	10	150	80	9.7	>5	24, 55, 160
1815/4	53	6	<10	30	8.3	>5	Full profile
1056/5	10	57	413	79	9.3	>5	(24) (160)
1069/5	13	101	297	72	8.0	>5	24, (55), 160
1070/5	14	40	273	67	8.6	>5	24, (51), 55, (120), 160
1071/5	16	19	108	64	8.2	>5	24, 51, 55, 120, 160
1165/5	22	3	19	63	5.5	>5	24, 51, 55, 120, 160
1603/5	58	7	<10	21	ND	>5	Full profile
1960/5	84	<4	<10	12	16.1	ND	Full profile
2039/6	10	31	ND	75	0.5	1.7	NEG
2094/6	20	ND	ND	46	6.0	3.0	24, 51, 55, (64), (120), 160
2702/7	24	<4	ND	61	8.0	ND	Full profile
2784/7	31	15	ND	41	10.0	>5	Full profile
2799/8	19	4	ND	47	9.0	>5	24, 51, 55, 120
3343/8	54	21	ND	9	14.0	>5	Full profile
1049/8	258	10	ND	6	ND	>5	ND
1795/8	330	6	ND	5	ND	ND	ND
1888/9	28	6	ND	74	8.0	>5	24, 55, 120, 160
1986/9	79	10	ND	100	8.0	>5	24, (51), 55, 120, 160
2436/9	98	<4	ND	20	11.0	ND	Full profile

[a] The activities found in the RT assay are given as the amount (in picograms per milliliter) of the reference rRT that gives equal activity. Positivity in the RT assay is set at ≥4 pg/ml (and having a corresponding signal of at least two times the background signal). For the RTb-Ab assay, results are expressed as the percentage relative to a 100% RT activity control incubated with sample dilution buffer, >50%, negative for HIV-1 RTb-Ab, ≤50%, positive for HIV-1 RTb-Ab.

[b] Cutoff for assay, <10 pg/ml, negative; >10 pg/ml positive.

[c] Cutoffs for assays are defined as absorbance per cutoff; ≥1, positive; <1, negative.

[d] ND, not determined.

[e] NEG, negative.

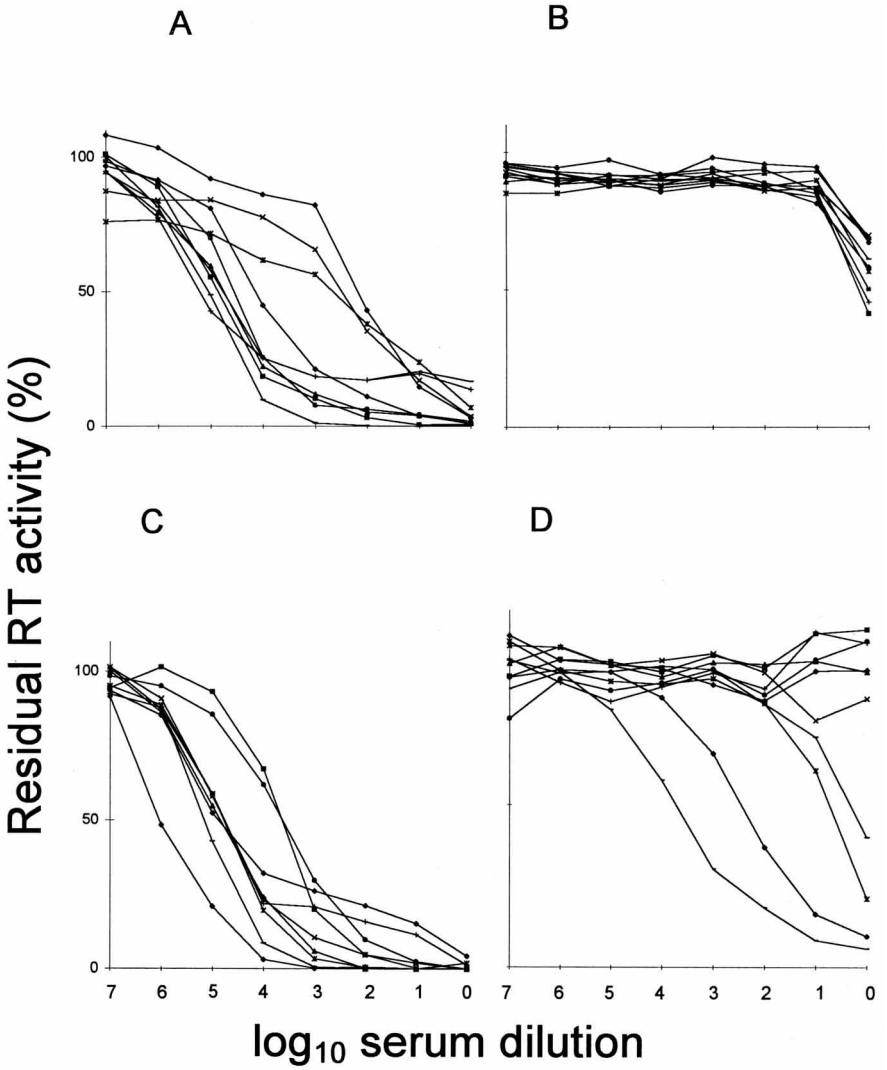

FIG. 6. The cross-reactivity between HIV-1 and HIV-2 RTb-Abs and their homologous RTs. The assays were performed by using standard procedure for RTb-Ab endpoint titer determination outlined in Materials and Methods, except for the inclusion of HIV-2 RT as the source of RT for HIV-2 RTb-Ab determination and cross-reactivity with HIV-1 sera. (A) HIV-1-positive sera with HIV-1 RT. (B) HIV-1-positive sera with HIV-2 RT. (C) HIV-2-positive sera with HIV-2 RT. (D) HIV-2-positive sera with HIV-1 RT.

sample was also classified as being WB negative, as defined by the WHO criteria for WB positivity (28). All African HIV-1-positive serum samples diagnosed by standard HIV Ab assays and WB were positive for RTb-Ab. Three serum samples from the African panel of sera screened positive for RTb-Ab but were defined as negative by standard techniques. Interestingly, two of these individuals posed diagnostic problems by conventional assays. Both serum samples had indeterminate profiles by WB. One of the serum samples showed faint reactivity with the p51 region, and the other serum sample showed reactivity with the p51 and p55 regions, which, interestingly, are the bands for the RT Ab. One possibility is that they may be from individuals infected with unusual HIV-1 subtypes which may be missed by standard HIV Ab assays (12, 13, 18, 23, 25, 27).

The RTb-Ab assay had sensitivities of 99 and 100% with HIV-1 Ab-positive sera of Swedish and Tanzanian origin, respectively, and a specificity of 97.6% with HIV-1 Ab-negative sera from Tanzania. An earlier study with Swedish HIV-1 Ab-positive and Tanzanian HIV-1 Ab-negative sera gave sensitivities of 100% for both the Abbott IMx and Behringwerke Enzygnost assays and specificities of 98 and 99.7%, respectively (evaluation of nine commercially available combined anti-HIV-1 and anti-HIV-2 ELISAs, SIIDC, 1995). It must be stressed, however, that the Swedish panel of HIV-1-infected sera used in the current study was far from ideal for the determination of RTb-Ab because the sera had been frozen and thawed many times. We have shown that RTb-Ab titers are reduced upon freezing and thawing of serum more than five times (data not shown). Therefore, this process may have contributed to the higher residual RT activities found in the Swedish HIV-1 Ab-positive panel compared to those found in the African HIV-1 Ab-positive panel, which were much fresher.

The assay detected RTb-Ab in the sera of recently infected individuals within 1 month after the appearance of symptoms of primary infection. In the majority of sera RTb-Ab positivity was seen within 1 month, and in some sera positivity was seen as early as 10 to 12 days after primary infection symptoms. The

TABLE 2. Reproducibility data for the screening and endpoint titer determination of RTb-Ab[a]

Assay type, result, and serum sample no.	Within-assay % or titer						Between-assay % or titer		
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Mean	SD	CV (%)
Screening assay									
Negative									
1	103 (±5.0)	103 (±2.5)	106 (±2.0)	111 (±3.2)	110 (±5.6)	110 (±4.6)	107	3.4[b]	3.3
2	101 (±2.9)	97 (±4.0)	101 (±4.7)	90 (±6.3)	91 (±5.9)	98 (±5.2)	96	4.4[b]	4.6
3	106 (±4.0)	95 (±2.3)	95 (±3.9)	105 (±6.7)	103 (±5.8)	102 (±6.3)	101	4.4[b]	3.2
4	92 (±3.6)	90 (±2.4)	95 (±2.9)	98 (±6.7)	89 (±5.3)	95 (±3.2)	93	3.0[b]	3.2
Positive sera									
1	11 (±1.4)	11 (±2.4)	9 (±0.5)	11 (±1.0)	9 (±0.9)	9 (±1.2)	10	0.98[a]	9.8
2	20 (±1.5)	20 (±3.1)	21 (±3.3)	22 (±1.2)	17 (±0.5)	23 (±1.9)	21	1.95[a]	9.4
3	14 (±1.8)	17 (±1.6)	18 (±1.1)	12 (±1.0)	12 (±1.2)	13 (±1.0)	14	2.54[a]	18.1
4	5 (±2.2)	5 (±2.1)	6 (±1.8)	4 (±0.8)	5 (±1.1)	5 (±0.5)	5	0.27[a]	5.4
Titer determination assay, positive sera									
1	4,248	4,995	4,264	4,315	4,704	4,214	4,457	320	7.2
2	27	25	22	21	24	29	25	3.0	12.1
3	22,284	21,681	19,136	18,059	19,102	18,886	19,858	1,701	8.5
4	502	491	475	454	486	426	472	28	5.8

[a] All sera were tested on six independent occasions (runs 1 to 6). For the screening assays each serum sample was tested six times in each run, after which the within-assay mean and SD were calculated. For the titer determination assay each serum sample was tested once in each run. Results for screening assays are percent (SD). Results for titer determination assay are titers.

[b] Between-assay SDs for the screening assays were calculated by using the modified SD formula, $[(\text{SD of the assay means})^2 - (\text{mean within-assay SD})^2/r)]^{1/2}$, where r is the number of replicates (2). The SD achieved was then used to calculate the CV by using the formula CV = (SD/mean) × 100. The standard SD formula for single samples was used to calculate the SD in the titer determination assay. The SD achieved was used to calculate the CV by using the formula defined above.

assay showed a direct correlation with the appearance of the p51 band on Western blots, therefore indicating at least the same level of sensitivity as WB. Antibodies to HIV-1 *env* peptides (gp160 and gp120) are usually the first to appear by WB after HIV-1 infection (Table 1). Therefore, HIV-1 RTb-Ab detection is normally not the optimal test for the early detection of HIV-1 infection in recently exposed individuals. However, our group has detected RTb-Ab in some serum samples prior to the detection of anti-gp160 and anti-gp120 Abs in an earlier study (21). This may also be the case for the three Tanzanian serum samples which were positive for RTb-Ab but which were negative by standard methods. RT activity in the patient's sera peaked at the same time (days 8 to 13) that p24 antigen levels peaked. RT activity, however, was still detected in small amounts after the induction of HIV-1 RTb-Ab. This is probably due to activities of RTs other than HIV-1 RT present in the patient sera. We have earlier seen that RT activity can be detected in HIV-negative sera at a level corresponding to 1 to 4 pg of the kit recombinant HIV-1 RT per ml. In general, RTb-Ab positivity was established 4 to 10 days after the peak level of viremia. However, decreasing residual RT activity was often seen prior to RTb-Ab positivity, as defined in Materials and Methods. In parallel with the RT assay used in the study, the RTb-Ab assay provides a simple and sensitive approach for the monitoring of individuals suspected of recent exposure to HIV-1, because a direct correlation between serum RT activity during HIV-1 viremia followed by the appearance of RTb-Ab can be easily measured.

The assay had a low level of false positivity, even when used to screen sera which were specifically selected for their ability to give false-positive results in other HIV Ab assays. Of 91 problem serum samples tested, only 2 gave weakly positive results by the RTb-Ab assay. The majority of sera in this panel gave residual RT activities of between 80 and 110%, indicating that interfering factors found in the sera which caused false-positive results in the other tests did not interfere with the RTb-Ab assay. HIV-1 RTb-Ab was also shown to be highly type specific. Of the 10 HIV-1 Ab-positive serum samples titrated toward HIV-2 RT, none displayed any tendency for cross-reactivity. The small inhibition of the HIV-2 RT seen in Fig. 6B in the highest concentration of HIV-1 Ab-positive serum is due to disturbing factors in the serum; these factors can include RNases, DNases, proteases, nucleotidases, nucleotides, and competing template-primers present in the serum sample and/or in the cell culture supernatant if the RT used is cell culture derived. The assay is calibrated for optimal HIV-1 RTb-Ab detection by using defined amounts of serum which do not normally disturb the HIV-1 rRT used unless blocking Ab is present. The HIV-2 RT used was more sensitive to serum inhibition than the HIV-1 rRT (Fig. 6B). This effect of minor inhibition at the highest concentration of serum was also seen when the HIV-2 RT was titrated against HIV-negative sera (data not shown). One explanation for this process is that the HIV-2 RT was cell culture derived and therefore the HIV-2 system contained more disturbing factors than the HIV-1 system, which used HIV-1 rRT. In contrast, HIV-2 antibody-positive sera showed some degree of cross-reactivity toward HIV-1 RT; however, for three of the four cross-reacting serum samples, a 3,000 times lower titer to HIV-1 RT than toward the homologous RT was obtained. The remaining serum sample had RTb-Ab titers toward HIV-1 and HIV-2 RTs of equal magnitude. This serum sample is quite unusual because it was positive for both HIV-1 and HIV-2 by WB. The serum was later diagnosed by a discriminatory peptide assay as being HIV-2 infected. The various degrees of cross-reactivity between HIV-2 RTb-Ab and HIV-1 RT indicate a lower type specificity for the HIV-2 RTb-Ab.

The assay is highly versatile in that it can be easily adapted for the measurement of RTs and RTb-Abs from other retroviruses. Provided that the RT is available and the correct

reaction solution is characterized, Ab blocking the RT can be detected. As a result, our group has measured RT activity during primary infection in SIV-infected macaque, followed by the appearance of RTb-Ab over the entire course of SIV infection, from acute infection to terminal stages of disease (8). We have also shown the RT assay's ability to detect viral replication in cell cultures of HIV-2, whereas it was shown that p24 assays cannot (7). The RT assay is also being optimized for the detection of Moloney murine leukemia virus and endogenous retroviral RT activity (work in progress).

The assay measures a *pol* gene product Ab (RTb-Ab). Most vaccines in field trials are based on gp160, gp120, and gp41 peptides (*env* gene products). Therefore, standard HIV-1 Ab ELISAs which detect gp160, gp120, and gp41 Ab cannot be used as follow-up for HIV-1 infection in individuals participating in trials. Thus, one important application of the assay could be the determination of HIV-1 infection in vaccine trials with these vaccines. The assay can also be used to detect infection in subjects in other types of vaccine trials, so long as the vaccines are devoid of the *pol* gene. It is also worth mentioning that the *pol* gene domain which forms the catalytic center of RT is conserved among all categories of RTs on the basis of amino acid sequence comparisons (14). With the emergence of new HIV-1 subtypes which do not belong to standard typing criteria (types A to H) (4, 11) and the highly variable *env* and *gag* gene products found between different isolates, it is perhaps only a matter of time before there emerge more HIV-1 strains which evoke immune responses which are not detected by standard HIV Ab assays based on recombinant or synthetic *env* and *gag* gene peptides from old laboratory isolates (13, 18, 25, 27). The catalytic site of the *pol* gene product (RT) probably remains relatively conserved among all HIV-1 subtypes. Therefore, antibodies developed in response to these epitopes will probably be relatively uniform among individuals infected with different subtypes of HIV-1. If this is the case, the emergence of new strains of virus that may be missed by standard HIV Ab assays could possibly be detected by the RTb-Ab assay (work in progress).

In conclusion, the RTb-Ab assay was shown to be highly sensitive and specific. The assay is simple to operate, uses nonradioactive product detection, and is compatible with all laboratories performing routine ELISA techniques. Together with the RT assay, the RTb-Ab assay provides an adequate means for monitoring individuals suspected of recent HIV-1 infection. Recently, an indication for the use of three antibody assay techniques against three different viral epitopes instead of the more conventional WB assay has been proposed to verify positive results (20). The RTb-Ab assay is the only standardized commercially available assay for the measurement of the HIV-1 *pol* gene product Ab and therefore would be highly suitable for this purpose. The assay could also be a useful tool for diagnosing HIV-1 infection in individuals participating in vaccine trials with vaccines devoid of the *pol* gene product, because it provides a simpler and more economical technique than PCR. As a final remark, the conserved nature of the HIV RT may evoke immune responses (i.e., RTb-Ab) which are uniformly inhibitory to all subtypes of HIV-1 RT (work in progress).

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