Comparison of an Assay Using Signal Amplification of the Heat-Dissociated p24 Antigen with the Roche Monitor Human Immunodeficiency Virus RNA Assay

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We compared an assay using signal amplification of a heat-dissociated p24 antigen (HDAg) with the Roche Monitor human immunodeficiency virus (HIV) RNA assay. The two assays gave comparable results when 130 specimens from 130 patients were tested ($r = 0.60$, $P < 0.0001$). The HDAg assay was almost as sensitive (85%) as the Roche HIV RNA kit (95%), just as specific (25 negative results from 25 HIV seronegative volunteers [100%]), less variable (mean log standard deviation of 0.07 compared to 0.11 when eight specimens were tested three or four times), and less expensive (reagent and labor costs, \$8 versus \$75). The assay appeared to be useful for monitoring established patients ($n = 17$) and identifying seroconverters ($n = 4$). HIV subtypes A to F were all recognized. This assay should be useful for monitoring patients in resource-poor countries and for monitoring vaccine recipients.

Although they are sensitive, human immunodeficiency virus type 1 (HIV-1) RNA assays are expensive and somewhat variable, and they require costly equipment and considerable technical expertise. A “low-tech” assay with the sensitivity of an RNA assay but with the reproducibility and cost of a p24 antigen assay would be of benefit, especially for monitoring patients in resource poor countries and in monitoring HIV vaccine recipients for possible infection.

Such an assay has been described by Jorg Schupbach and colleagues in a series of papers (1, 2, 8, 11, 14–17). The method involves dissociating the antigen-antibody immune complexes circulating in HIV-seropositive individuals by boiling and then using signal amplification with biotinyl-tyramide and streptavidin-horseradish peroxidase. The linear range of the assay has been increased by quantitative kinetic enzyme-linked immunosorbent assay (ELISA) reader software developed by O. E. Varnier and M. Giacommi (5). Despite the many advantages of this procedure, to date, only a limited number of additional laboratories have reported using this assay successfully (6, 9).

Since this assay is less complex technically, less variable, and considerably less expensive than conventional HIV RNA assays, we wanted to see if it would be suitable for use in developing countries. Consequently, we evaluated the assay by performing a comparison with the Roche Monitor HIV RNA assay. In particular, we were interested in determining whether the assay could be used with HIV subtypes other than B.

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MATERIALS AND METHODS

Specimens. Excess plasma from 130 HIV-seropositive patients that remained after determining HIV RNA levels by the Roche Monitor assay ($n = 112$) or the Organon Teknica (Durham, N.C.) NucliSens assay ($n = 18$) was tested with an assay using a heat-dissociated, “boosted” p24 antigen (HDAg). In addition, EDTA plasmas from 25 seronegative hospital workers were tested in both assays. A panel of viral load standards for HIV subtypes A to F were a generous gift from Merlin Robb of Walter Reed Army Institute of Research (Rockville, Md.) (10). Seroconversion panels (PRB 923, PRB 939E, PRB 944, and PRB 935) were purchased from Boston Biomedica, Inc (West Bridgewater, Mass.).

HDAg assay. For the HDAg assay, we used the method described by Nadal et al. (11). Briefly, plasma was diluted 1:6 in 0.5% Triton X-100 and heated at 100°C for 5 min in a dry heat block. Treated plasma (0.25 ml) was transferred to wells of a HIV-1 p24 core profile ELISA kit (DuPont, Wilmington, Del.), covered, and incubated 2 h at room temperature on a microtiter plate shaker. Wells were washed with 1× wash buffer, 0.1 ml of biotinylated detector antibody was added, and the mixture was incubated for 1 h at 37°C. After a washing, 0.1 ml of streptavidin-horseradish peroxidase solution was added to the wells, and the plate was incubated for 15 min at 37°C (Renaissance ELAST ELISA amplification system; DuPont). After a washing, 0.1 ml of biotinyl-tyramide solution was added to the wells, and the plate was incubated for 15 min at room temperature. Following a washing, 0.1 ml of streptavidin-horseradish peroxidase diluted in washing buffer to which had been added 1% bovine serum albumin was added to the wells, and the plate was incubated for 15 min at room temperature. *O*-phenylenediamine substrate solution (0.1 ml) was added to the wells after a washing, and the plate was inserted into a kinetic ELISA reader (Molecular Devices, Palo Alto, Calif.). By using Quanti-Kin detection system software (DL3; Diagnostica Ligure s.r.l., Genoa, Italy) (7), kinetic readings were performed during the initial 10 min of incubation. At the end of 30 min, the colorimetric reaction was stopped by the addition of 0.1 ml of stop solution, and the end-point reading was taken as indicated by the Quanti-Kin software. For quantitation, a cutoff corresponding to the mean plus 3 standard deviations was calculated by the software. We did not routinely go back and retest specimens that were close to the cutoff. However, most specimens were tested in duplicate and repeated values were very close, especially at lower concentrations.

HIV RNA assay. The standard Roche Amplicor Monitor assay (version 1.0) using 0.2 ml of plasma and a limit of quantitation of 400 copies/ml was used to measure HIV RNA levels, except for the testing of plasma specimens from

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FIG. 1. Comparison of HIV RNA (Roche 1.0) and the HDAg assay. Circles, 112 plasma specimens from 112 U.S. patients with subtype B; squares, 18 specimens from 18 Malawian patients with subtype C.

Malawi (12). In that case, the NucliSens HIV-1 QT RNA assay, which has a lower limit of quantitation of 400 copies/ml, was used. Manufacturers' instructions were followed for both RNA assays (12).

Statistical analysis. HDAg and HIV RNA concentrations were $\log_{10}$ transformed for all analyses. SAS 8.01 was used. Paired *t* tests and Pearson's correlation coefficient were used to compare results of the two assays.

RESULTS

Sensitivity and specificity. A total of 130 plasma specimens from 130 patients were tested in each assay; 112 were from patients from the United States and were presumed to be subtype B, and 18 were subtype C and were obtained from patients in Malawi (Fig. 1). Overall concordance was 84.6% (110 of 130), with 82.3% of the specimens being positive in both assays and 2.3% being negative in both assays. The RNA assay was more sensitive, yielding 124 positive results (95.4%), compared with 110 positive HDAg results (84.6%). In terms of viral load, the HDAg assay detected 71.4% (5 of 7) of specimens with a viral load of $<1,000$ RNA copies/ml, 65% (13 of 20) of specimens with viral load between 1,000 and 10,000 copies/ml, 87% (47 of 54) of specimens with a viral load between 10,000 and 100,000 copies/ml and 97.7% (42 of 43) of specimens with a viral load of $>100,000$ copies/ml. The two assays were significantly correlated ($r = 0.596$, $P < 0.0001$) (Fig. 1). We tested plasma from 25 HIV-seronegative workers and obtained no positive results (100% specificity for both assays).

Linearity. Tenfold dilutions of three HIV culture supernatants were tested in the HDAg assay (Fig. 2). The linear dynamic range extended from at least 4×10^6 to 4×10^3 fg/ml.

Intra-assay variation. Six specimens were tested three or four times in each assay to assess intra-assay variability (Table 1). The overall mean log standard deviation was 0.11 for the Roche assay (range, 0.07 to 0.20) while the mean log standard deviation for the boosted p24 antigen assay was 0.07 (range, 0.03 to 0.12).

FIG. 2. Linearity of the HDAg assay when three culture supernatants were diluted and tested.

Patient monitoring. Seventeen patients initiating new antiretroviral therapy (ART) were tested in the two assays. Five to eleven specimens from each individual were tested. Representative results from the two patients with the most data points are shown in Fig. 3. In 13 of 17 cases the HDAg completely paralleled the RNA results. In the remaining 4 cases, there was a single time point for each subject with a discrepant result; i.e., p24 antigen increased while the HIV RNA decreased, or vice versa.

Seroconversion panels. Four seroconversion panels were tested. In three cases, HIV RNA became detectable 2 days before the appearance of standard p24 antigen or HDAg but 2 or more weeks prior to the appearance of antibodies to HIV. In one case (PRB 935), HDAg was the first to become positive, at 22 days prior to seroconversion, albeit at very low concentrations (172 and 181 fg/ml in repeated testing), followed by HIV RNA at 19 days (4,000 copies/ml according to data provided by Boston Biomedica) before seroconversion and lastly by the standard p24 antigen assay at 15 days before seroconversion. However, in this case, the day -19 specimen that was positive in the RNA assay was negative in the HDAg assay. The day -15 and day 0 (seroconversion) specimens were positive in all three assays. HDAg results were 18,654 and 17,220 fg/ml compared to 70,000 copies of HIV RNA/ml for the day -15 specimens and 24,063 and 24,807 fg/ml compared to 50,000 copies/ml for the day 0 samples. Specimens after seroconversion were available for one of the panels (PRB 923). Although the HIV RNA and HDAg results were positive at all time points, the standard p24 antigen assay became negative by the time of seroconversion or soon after the appearance of antibodies to HIV.

HIV subtypes. A panel of HIV isolates representing subtypes A to F (10) was tested in the assay (Table 2). In addition, plasma specimens from 18 HIV-positive patients from Malawi (all subtype C) were recognized (Fig. 1). In all cases, positive results were observed.

DISCUSSION

An estimated 90% of the HIV-infected people of the world live in the resource-poor countries of Africa, Asia, and Latin America (18). Since the International AIDS Conference held in Durban, South Africa, in July 2000, more attention has been paid to the epidemic in these areas. In the future, ART will be available to large segments of the populations of these countries. However, without therapeutic monitoring, physicians

TABLE 1. Intra-assay variation assessed by testing plasma from six individuals in triplicate or quadruplicate

Patient	RNA level				HDAg level				
	Log copies/ml			Log SD	Log fg/ml				Log SD
	Assay 1	Assay 2	Assay 3		Assay 1	Assay 2	Assay 3	Assay 4	
1	5.39	5.30	5.21	0.09	4.27	4.16	4.14	4.19	0.06
2	3.49	3.22	3.10	0.20	3.66	3.72	3.68	QNS ^a	0.03
3	4.15	4.07	4.21	0.07	4.88	4.71	4.71	4.92	0.11
4	4.10	4.29	4.26	0.10	4.33	4.30	4.29	4.36	0.03
5	5.34	5.41	5.52	0.09	4.96	4.94	4.98	4.72	0.12
6	4.98	4.87	5.08	0.11	4.86	4.94	4.92	4.81	0.05

^a QNS, quantity not sufficient.

may not be able to identify patients with virological failure, and this may limit the effectiveness of therapy and may promote the spread of drug resistance (13). The technical and financial requirements of performing routine monitoring of HIV levels of patients receiving ART by using molecular methods may simply be too much. The HDAg assay should provide a simpler and less expensive alternative.

Although somewhat less sensitive than the standard Roche RNA assay, the HDAg assay is still considerably more sensitive than either the standard p24 assay or even the acid-dissociated p24 antigen assay (14, 15). When 130 specimens were tested, the boosted p24 antigen assay detected 85%, compared with 95% of the same specimens in the RNA assay, without losing specificity. About two-thirds of specimens with HIV RNA levels less than 10,000 copies/ml and 87% or more of specimens with viral loads greater than 10,000 copies/ml were identified in the assay. The cost of the HDAg assay is approximately 1/10 the cost of an RNA assay.

Recent findings suggest that the sensitivity of the Roche ultrasensitive assay or even the standard Roche assay may not

be necessary. It has become increasingly clear that even combination ART will not eliminate HIV from the body (3, 7, 19). In addition, the numerous side effects and toxicities of the drugs have prompted new, less aggressive guidelines for the prescription of ART in adults and adolescents (www.hivatis.org). As a consequence the decreased sensitivity of the HDAg assay will probably not be as much of a liability as might have been perceived 2 or 3 years ago.

It is important to remember that p24 antigen assays and HIV RNA assays measure two different parameters of viral replication. Blood plasma HIV RNA assays quantitate virion-associated RNA, while p24 antigen can be either virion associated or relatively free, though it is largely bound in immune complexes (4). For this reason, it is probably not surprising that the assays do not give exactly the same results. However, Schup-

TABLE 2. Recognition of HIV subtypes in the HDAg assay

Walter Reed no.	Subtype	HIV RNA (log RNA copies/ml) ^a	HDAg (log fg/ml)
WR1	A	3.76	3.25
WR2	A	ND	3.18
WR3	A	ND	3.19
WR4	B	4.35	3.11
WR5	B	5.28	3.51
WR6	B	5.26	3.54
WR7	B	4.88	3.24
WR8	B	4.87	3.39
WR9	B	5.08	3.39
WR10	B	5.11	3.48
WR11	C	4.80	3.16
WR12	C	5.57	3.46
WR14	C	4.51	2.81
WR15	C	4.97	3.34
WR16	C	5.65	3.51
WR17	D	5.21	3.32
WR18	D	4.95	3.21
WR19	D	5.28	3.28
WR20	E	3.30	3.01
WR21	E	3.48	3.29
WR22	E	4.07	3.35
WR23	E	3.90	3.05
WR24	E	4.19	3.31
WR25	E	3.87	3.17
WR26	E	3.96	3.15
WR27	E	4.01	3.35
WR30	F	3.86	3.41
WR31	F	4.17	3.16
WR32	F	3.50	3.15

^a ND, not detected.

FIG. 3. Comparison of the Roche 1.0 HIV RNA results (squares) and HDAg assay (triangles) for patient monitoring. Panels A and B show results for two representative patients.

bach and colleagues have argued that viral proteins are more commonly associated with mechanisms of viral pathogenesis than are viral nucleic acids and therefore should theoretically be better markers of disease progression (16).

For the first time we report that the HDag assay identified seroconverters only 2 days after the RNA assay and remained positive like the RNA assay while the standard p24 antigen assay was only transiently positive. This finding may be of benefit for people designing vaccine efficacy trials. Standard antibody ELISAs will not be useful with many vaccinees, since one would hope that all recipients would mount some sort of antibody response. Screening large numbers of recipients periodically by RNA testing will be very expensive, even if a qualitative assay is used. The standard p24 antigen assay lacks sensitivity and is usually positive for only a few weeks around the time of infection. However, the sensitive yet inexpensive HDag assay, with its ability to recognize subtypes A to F as well as HIV-1 group O (2), is an obvious choice for those conducting vaccine trials. An additional benefit is its ability to be automated. We believe that this assay will prove useful for monitoring patients in resource-poor countries and will also be helpful in monitoring HIV vaccine recipients.

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REFERENCES

1. Boni, J., O. Opravil, Z. Tomasik, M. Rothen, L. Bisset, P. J. Grob, R. Luthy, and J. Schupbach. 1997. Simple monitoring of antiretroviral therapy with a signal-amplification-booster HIV-1 p24 antigen assay with heat-denatured plasma. *AIDS* 11:F47-F52.
2. Burgisser, P. P. Vernazza, M. Flepp, J. Boni, Z. Tomasik, U. Hummel, G. Pantaleo, and J. Schupbach. 2000. Performance of five different assays for the quantification of viral load in subjects infected with various subtypes of HIV-1. *J. Acquir. Immune Defic. Syndr.* 23:138-144.
3. Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 278:1295-1300.
4. Fiscus, S. A., J. D. Folds, and C. M. van der Horst. 1993. Infectious immune complexes in HIV-1-infected patients. *Viral Immunol.* 6:135-141.
5. Giacomini, M., J. L. McDermott, A. A. Giri, I. Martini, F. B. Lillo, and O. E. Varnier. 1998. A novel and innovative quantitative kinetic software for virologic colorimetric assays. *J. Virol. Methods* 73:201-209.
6. Goldschmidt, P. L., A. Devillechabrolle, Z. Ai-Arkoub, and J. T. Aubin. 1998. Comparison of an amplified enzyme-linked immunosorbent assay with procedures based on molecular biology for assessing human immunodeficiency virus type 1 viral load. *Clin. Diagn. Lab. Immunol.* 5:513-518.
7. Gunthard, H. F., D. V. Havlir, S. Fiscus, Z.-C. Zhang, J. Eron, J. Mellors, R. Gulick, S. D. W. Frost, A. J. L. Brown, W. Schleif, F. Valentine, L. Jonas, A. Meibohm, C. C. Ignacio, R. Isaacs, R. Gamagami, E. Emini, A. Haase, D. D. Richman, and J. K. Wong. 2001. Residual human immunodeficiency virus (HIV) type 1 RNA and DNA in lymph nodes and HIV RNA in genital secretions and in cerebrospinal fluid after suppression of viremia for 2 years. *J. Infect. Dis.* 183:1318-1327.
8. Ledergerber, B., M. Flepp, J. Boni, Z. Tomasik, R. W. Cone, R. Luthy, and J. Schupbach. 2000. Human immunodeficiency virus type 1 p24 concentration measured by boosted ELISA of heat-denatured plasma correlates with decline in CD4 cells, progression to AIDS, and survival: comparison with viral RNA measurement. *J. Infect. Dis.* 181:1280-1288.
9. Lyamuya, E., U. Bredberg-Raden, A. Massawe, E. Urassa, G. Kawo, G. Msemo, T. Kazimoto, A. Ostborn, K. Karlsson, F. Mhalu, and G. Biberfeld. 1996. Performance of a modified HIV-1 p24 antigen assay for early diagnosis of HIV-1 infection in infants and prediction of mother-to-infant transmission of HIV-1 in Dar es Salaam, Tanzania. *J. AIDS* 12:421-426.
10. Michael, N. L., S. A. Herman, S. Kwok, K. Dreyer, J. Wang, C. Christopher, J. P. Spadoro, K. K. Y. Young, V. Polonis, F. E. McCutchan, J. Carr, J. R. Mascola, L. L. Jagodzinski, and M. L. Robb. 1999. Development of calibrated viral load standards for group M subtypes of human immunodeficiency virus type 1 and performance of an improved Amplicor HIV-1 Monitor test with isolates of diverse subtypes. *J. Clin. Microbiol.* 37:2557-2563.
11. Nadal, D., J. Boni, C. Kind, O. E. Varnier, F. Steiner, Z. Tomasik, and J. Schupbach. 1999. Prospective evaluation of amplification boosted ELISA for heat-denatured p24 antigen for diagnosis and monitoring of pediatric human immunodeficiency virus type 1 infection. *J. Infect. Dis.* 180:1089-1095.
12. National Institute of Allergy and Infectious Diseases, Division of AIDS. 1997. DAIDS virology manual for HIV laboratories, publication no. NIH-97-3828. U.S. Department of Health and Human Services, Washington, D.C.
13. Pilcher, C. D., M. D. Perkins, S. A. Fiscus, D. M. Johnston, R. Diaz, U. H. Duque, A. M. Zago, F. Assad-Antunes, and J. J. Eron. 1999. Genotypic resistance and the treatment of HIV-1 infection in Espirito Santo, Brazil. *J. Infect. Dis.* 179:1259-1263.
14. Schupbach, J., J. Boni, Z. Tomasik, J. Jendis, R. Seger, C. Kind, and the Swiss Neonatal HIV Study Group. 1994. Sensitive detection and early prognostic significance of p24 antigen in heat-denatured plasma of human immunodeficiency virus type 1-infected infants. *J. Infect. Dis.* 170:318-324.
15. Schupbach, J., M. Flepp, D. Pontelli, Z. Tomasik, R. Luthy, and J. Boni. 1996. Heat-mediated immune complex dissociation and enzyme-linked immunosorbent assay signal amplification render p24 antigen detection in plasma as sensitive as HIV-1 RNA detection by polymerase chain reaction. *AIDS* 10:1085-1090.
16. Schupbach, J., Z. Tomasik, D. Nadal, B. Ledergerber, M. Flepp, M. Opravil, and J. Boni. 2000. Use of HIV-1 p24 as a sensitive, precise, and inexpensive marker for infection, disease progression and treatment failure. *Int. J. Antimicrob. Agents* 16:441-445.
17. Schupbach, J., J. Boni, M. Flepp, Z. Tomasik, H. Joller, and M. Opravil. 2001. Antiretroviral treatment monitoring with an improved HIV-1 p24 antigen test: an inexpensive alternative to tests for viral RNA. *J. Med. Virol.* 65:225-231.
18. UNAIDS. 2000. Report on the global HIV/AIDS epidemic. UNAIDS, Geneva, Switzerland.
19. Wong, J. K., M. Hezareh, H. F. Gunthard, D. V. Havlir, C. C. Ignacio, C. A. Spina, and D. D. Richman. 1997. Recovery of replication-competent G+HIV despite prolonged suppression of plasma viremia. *Science* 278:1291-1295.