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Viral RNA and p24 Antigen as Markers of HIV Disease and Antiretroviral Treatment Success

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Key Words
HIV infection · Viral load · HIV RNA · p24 antigen · Antiretroviral treatment monitoring

Abstract
HIV-1 RNA has become the standard for monitoring antiretroviral therapies. Dogma predicts, however, that a viral protein like p24 should be at least as good a marker of HIV disease activity, provided that it is measured with sufficient sensitivity and accuracy. Simple modifications including use of a more efficient virus lysis buffer, heat-mediated destruction of antibodies interfering with antigen detection, and tyramide signal amplification for increased sensitivity have highly improved the HIV-1 p24 antigen assay. The p24 antigen assay is inferior to RT-PCR in detecting viral particles, but the presence of extraviral p24 antigen in most samples makes largely up for this. p24 antigen testing is similarly sensitive and specific in diagnosing pediatric HIV infection, in predicting CD4+ T cell decline and clinical progression at early and late stage of infection, and suitable for antiretroviral treatment monitoring in both adults and children. Notably, p24 antigen was measurable even in patients with stably suppressed viremia, and its concentrations were correlated negatively with the concentrations of CD4+ T cells and positively with the concentrations of activated CD8+ T cell subsets. p24 antigen is an excellent marker of HIV expression and disease activity and can be used in the same fields of application as HIV RNA is used. The test is validated for subtype B, but requires further studies for non-B subtypes.

The demonstration that drugs that block HIV replication can halt and even partially reverse the progression of HIV-infected persons towards destruction of the immune system, AIDS and death [1–9] and that discontinuation of antiretroviral treatment (ART), or viral mutation leading to loss of its efficacy, is followed by a rapid rebound of viral RNA in plasma and renewed loss of CD4+ T lymphocytes [10, 11] are clear proof of the concept that the amount of the viral pathogen in an infected person, the so-called viral load, determines disease outcome.

Favored by the development of highly efficient amplification techniques such as polymerase chain reaction (PCR), procedures for quantifying viral nucleic acids, in particular the viral RNA in plasma, have become standard tools for viral load assessment. It has been demonstrated that the concentration of HIV RNA in plasma is predictive of CD4+ T cell decline, progression to clinical AIDS and survival [10, 12–15]. Consequently, HIV RNA in plasma has become a major endpoint parameter for clinical evaluation of ART regimens and for monitoring therapy in individual patients [16–18].

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The dogma that the molecular mechanisms of viral pathogenesis are mainly based on viral proteins remains, however, unrefuted despite the impressive advancements in nucleic acid-based tests, and it predicts that a viral protein should be as good a marker of disease activity as is the viral RNA, provided that it can be measured with sufficient sensitivity and accuracy. Some early studies investigating patients soon after seroconversion indeed have reported that detectability of p24 antigen was a stronger predictor of progression to AIDS than was HIV-1 RNA concentration [19, 20], but all studies performed at that time showed less frequent detection of p24 antigen than of HIV-1 RNA, demonstrating a true problem of sensitivity [19–22]. During the past decade the antigen test has been greatly improved, however, and sufficient data have now accumulated to justify reassessment of antigen testing in HIV disease.

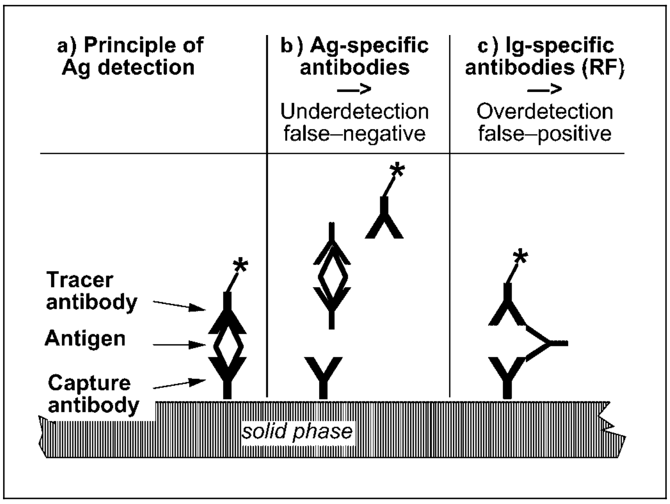

Fig. 1. Principle of antigen testing and interference by antigen-specific or Ig-specific antibodies (for explanation see text) [with permission, 67].

Principle and Problems of Antigen Detection

The test principle consists of binding the p24 antigen present in a sample to p24-specific, mono- or polyclonal 'capture' antibodies coated onto a solid support. Unbound sample components are washed away, and bound antigen is detected with another p24-specific 'tracer' antibody to which an enzyme (horseradish peroxidase or alkaline phosphatase) is conjugated capable of signal generation when combined with a suitable substrate (fig. 1a). For confirmation of a reactive diagnostic result, the sample must be subjected to a neutralization assay. This means that the antigen test is repeated in the presence of high-titered HIV-specific antibodies. These bind the antigen in immune complexes, thus preventing its detection in the test (fig. 1b).

This test system is frequently confronted with three problems. One is the presence of p24-specific antibodies, which as in the neutralization assay immune-complex the antigen, thus causing underdetection or false-negative results [23–25]. A second problem is the presence of immunoglobulin-specific, rheumatoid-factor-like antibodies which may bridge the capture and the tracer antibodies of an antigen test and thus cause overdetection or false-positive results (fig. 1c). This type of problem may be present when in the neutralization test the addition of HIV-specific antibodies to the test sample does not result in a higher degree of signal reduction than does the addition of antibodies from an HIV-negative control. A third problem is the low sensitivity of the test compared to nucleic acid-based methods [26].

How to Improve p24 Antigen Tests

Improvements introduced into p24 antigen testing were primarily aimed at improving detection of immune-complexed antigen. Acidification or base treatment leads to a significant, though incomplete, release of antigen, thus increasing the proportion of antigen-positives significantly [27]. Experience shows, however, that a considerable part of antigen cannot be freed from complexes or reassociates again when the pH of the sample is neutralized in order to allow binding of the antigen to the capture antibody. In addition, these treatments will release rheumatoid factors from preformed immunoglobulin-anti-immunoglobulin complexes, thus aggravating the problem of overdetection or false positivity [28]. The combination of these two effects whose extent in a given sample cannot be predicted prevents an accurate measurement of the true concentration of p24 antigen in a sample.

Heat Denaturation Eliminates Antibody Interference

Interference by antibodies (problems 1 and 2) can be eliminated efficiently by heat-mediated destruction of the three-dimensional structure of antibodies. Boiling the diluted sample for 5 min abolishes all antigen binding by antibodies, but leaves the p24 antigen reactive in tests that feature reagents (mono- or polyclonal antibodies for capturing and tracing) which recognize heat-denatured antigen. This effect has been demonstrated in numerous experiments involving both artificial immune complexes

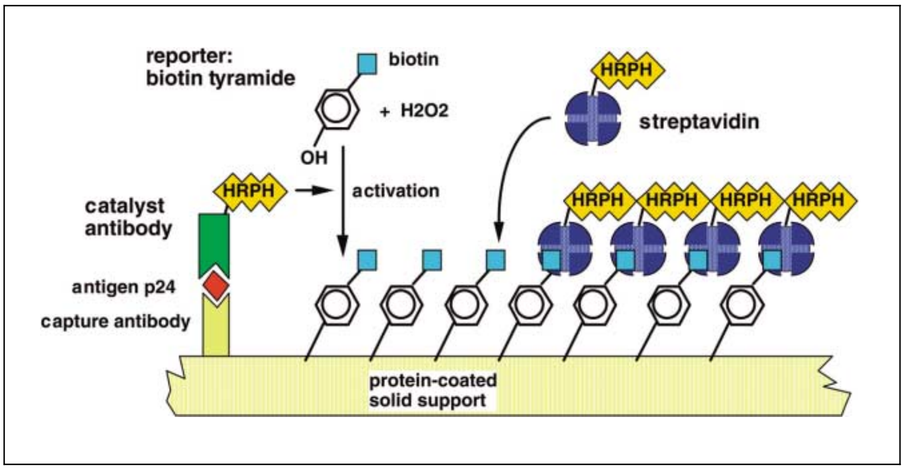

Fig. 2. Principle of tyramide-mediated signal amplification of ELISA [34]. The tracer antibody which is labeled with horseradish peroxidase H (HRPH) is used as a catalyst antibody for the activation of the biotin tyramide reporter molecule. The activated reporter binds to tyrosine residues of any immobilized protein. Added HRPH-labeled streptavidin thus finds a highly increased number of targets, thereby generating an enhanced signal [with permission, 68].

Table 1. Virus component detection by signal-amplification-boosted p24 antigen ELISA of heat-denatured plasma and PCR for HIV-1 RNA [with permission, 35]

Classification	p24 antigen ELISA		HIV-1 Monitor version 1.0	
	positive/tested	%	positive/tested	%
By CDC 93 category				
A	71[a]/74	95.9	46/50	92
B	50[a]/51	98.0	31/32	96.9
C	57/57	100	35/35	100
By CD4+ cell category				
1 (≥500/μl)	12/14	85.7	6/6	100
2 (200–499/μl)	52[a]/53	98.1	34/38	89.5
3 (<200/μl)	114[a]/115	99.1	72/73	98.6
Total	178/182	97.8	112/117	95.7

[a] After subtraction of one reactive sample not confirmed by neutralization.

and natural patient samples [29–31]. Thus, this simple measurement permits to determine a sample's true antigen content.

The practical value of this first heat-denaturation-based procedure was established by a study of children born to HIV-1-infected mothers. Due to transplacentar transport of maternal IgG such children have usually high concentrations of HIV-specific IgG antibodies, resulting in immune complexation of all p24 antigen. In this retrospective study the procedure's specificity in 390 samples from uninfected children born to HIV-positive mothers was 96.9% after initial testing and 100% after neutralization. Diagnostic sensitivity among 125 samples from infected children was, at a detection limit of 2 pg/ml, 96.0% (97% of which neutralizable) compared with 47.7% for regular antigen (76% neutralizable), 96% for PCR for HIV-1 DNA, and 77% for virus culture [32]. The study also found low levels of p24 antigen in 29% of cord blood sera, a postnatal increase to levels that were during the first 6 months of life – i.e., the time of the primary infection – inversely correlated with survival, and persistence of antigenemia in all children thereafter. These findings were in perfect agreement with the later demonstration by others that high viral RNA levels at birth and during primary viremia were associated with early onset of symptoms and rapid disease progression [33].

Increase of Sensitivity by Tyramide Signal Amplification

Despite its high diagnostic sensitivity in pediatric HIV infection the procedure was not sufficiently sensitive, as shown by the fact that only 22% of the mothers of these children tested positive [32]. The antigen assay was therefore boosted by the simple, commercially available tyram-

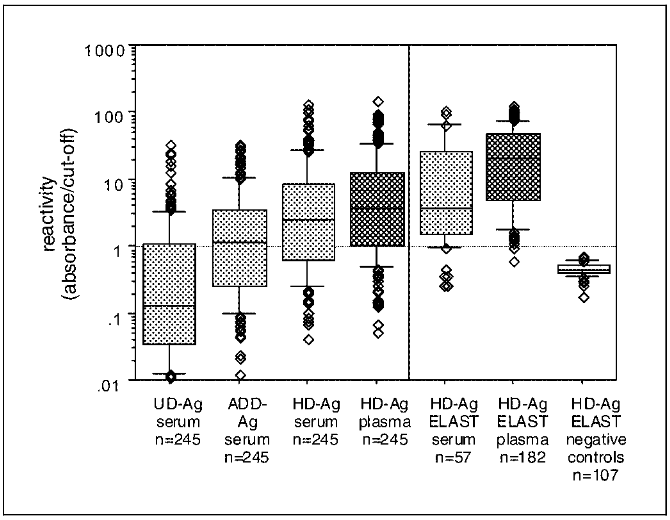

Fig. 3. Overview on the effects achieved by the various measures used to improve antigen detection. The box plot rendition of the reactivity of each sample is a percentile-based analysis, in which the five horizontal lines represent, from bottom to top, the 10th, 25th, 50th, 75th and respectively 90th percentile and outrunners are plotted individually. UD-Ag = Undenatured antigen; ADD-Ag = antigen after acid disruption of immune complexes; HD-Ag = heat-denatured antigen; HD-Ag ELAST = heat-denatured antigen combined with detection by ELAST tyramide signal amplification boosted ELISA [with permission, 35].

ide signal amplification system whose principle is shown in figure 2 [34]. A comparison of paired serum and plasma samples from 245 adult HIV-1-infected individuals of all stages of chronic infection furthermore showed that plasma contains more p24 antigen than serum (fig. 3). In combination, heat denaturation, use of plasma instead of serum and tyramide signal amplification led to a procedure that had the same diagnostic sensitivy as the Roche Amplicor HIV-1 Monitor® version 1.0 which had a detection limit of 200–400 HIV-1 RNA copies/ml in our hands (table 1) [35].

Further Improved Antigen Detection by a Better Virus Lysis Buffer

Since we discovered that certain samples with HIV RNA concentrations that should have permitted detection of the particle-associated antigen were negative in the assay we replaced the Triton X-100 buffer of the kit by one containing a mixture of different detergents [36]. Pretreatment of samples with this buffer results in significantly improved detection of particle-associated antigen, as also found by others [37].

p24 and HIV RNA Are Related, but Different Viral Markers

The production and release of p24 and particle-associated RNA are biologically tightly linked. They are both derived from unspliced viral mRNA, and p24 is a component of the viral protein precursors $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$, thus being stoechiometrically linked with another precursor component, the nucleocapsid p9, which is directly involved in encapsidation of viral RNA into the particles. p24 is an important structural component of the retroviral particle and estimated to be present at 2,000–4,000 molecules in each virion [38]. It is clear that increased viral transcription will normally lead to increased intracellular concentrations of both genomic RNA and viral proteins; this in turn will be followed by increased particle formation and release, leading to increased extracellular concentrations of the two markers. On the other hand, destruction of virus-producing cells by viral or immune cytopathicity will increase the extracellular concentrations of viral proteins, while not leading to a likewise increased concentration of HIV RNA. Similarly, destruction of virus particles should lead to instant degradation of the enclosed viral RNA by RNases present at high concentrations in all body compartments, while the enclosed viral proteins should be more resistant and thus persist outside the particle. In support of this we have been able to measure p24 antigen in 92.5% of serum samples stored for 10 years and found the p24 concentrations to be significantly correlated with the risk of progression to AIDS, while HIV-1 RNA was degraded to undetectable levels in more than 70% of the samples. Thus, although we expect an overall positive correlation of the concentrations of HIV RNA and viral protein, e.g. the p24 antigen, which indeed has been found in all published comparisons [35, 39–45], there are situations in which a positive correlation cannot be anticipated.

Viral RNA and p24 Antigen during the Natural Course of the HIV Infection

Figure 4 summarizes the course of HIV RNA, p24 antigen and immunological markers during HIV infection. In acute infection, replication of HIV within the lymphatics, which harbor 98% of the body's lymphocytes, causes in the absence of a specific immune response a rapid increase in the production and release of virus and virus-infected cells [12, 46–49]. Peak concentrations of viral RNA in plasma may vary widely, from 10^4 to more than 10^7 copies/ml [50, 51].

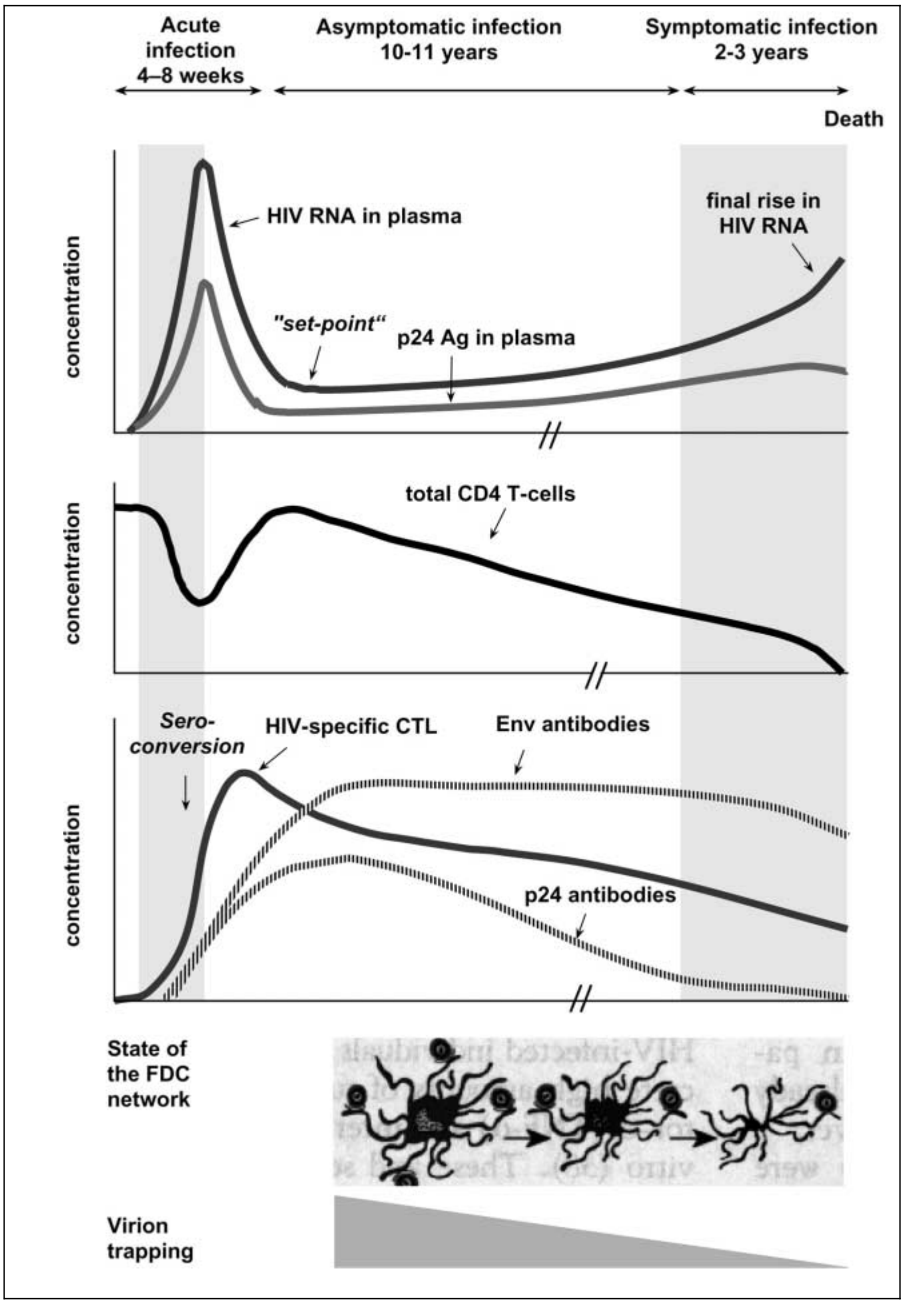

Fig. 4. Schematic overview of CD4+ T lymphocytes, HIV RNA, p24 antigen and immunological parameters in the course of the disease. Viral markers in plasma depend not only on production rates in the lymphoid tissues [also influenced by HIV-specific cytotoxic T lymphocyte (CTL) activity], but also on retention mechanisms exerted by an intact follicular dendritic network in combination with the humoral immune response. Note the difference in viral RNA and p24 antigen concentrations in final disease.

It is now clear that HIV RNA is the first viral marker detectable in acute infection. p24 antigen on average becomes positive 7 days after a HIV RNA test with a detection limit of 50 copies/ml. At the time of antigen conversion the concentration of viral RNA on average is 10,000 copies/ml [51]. As many as 5,000 virus particles are thus needed before the p24 antigen enclosed in these can be detected. Since there are no HIV-specific antibodies, there will be no immune-complexed p24 antigen.

Virus levels decrease with the onset of the antiviral immune response, namely, the production of HIV-specific cytotoxic T lymphocytes. Moreover, after seroconversion, antivirus antibodies that bind to virus particles and to which complement is fixed will increase virus retention on follicular dendritic cells of the lymphoid tissues. These cells, whose numerous processus form a dense network, carry complement receptors at high density and thus retain large quantities of immune-complexed infec-

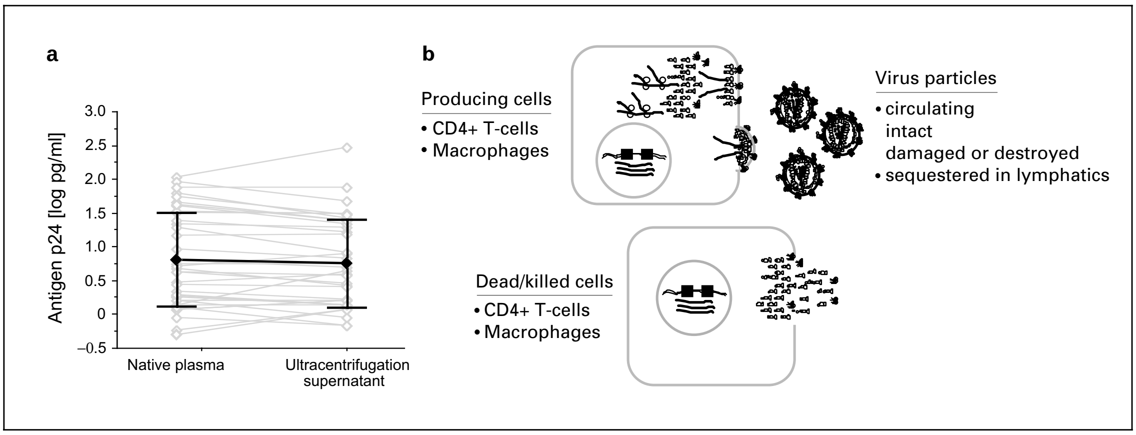

Fig. 5. p24 antigen in plasma originates from different sources. **a** Evidence for presence of p24 outside viral particles. Ultracentrifugation of plasma from patients in the chronic stage of HIV infection, while removing all viral RNA and reverse transcriptase activity (not shown), leaves most of the p24 antigen in the supernatant, thus indicating that most of the detectable antigen is not associated with viral particles. **b** Possible sources of p24 antigen and HIV-1 RNA in plasma. p24 antigen may originate from several sources including the structural protein of intact or defective viral particles present in the sample or released from particles degraded while entangled in the follicular dendritic cell network of the lymphatics. p24 antigen may also be released from HIV-producing cells or leak from cells killed either by viral or immune-mediated cytotoxicity. p24 antigen concentration in plasma may therefore be more representative of the total viral load in the body than is the HIV-1 RNA in plasma, which originates exclusively from intact circulating particles.

tious virions [52–54]. Since the half-life of virus particles is only a few hours [55], if not minutes [56], a large part of trapped virions will be degraded and their viral RNA digested. In agreement with this, the viral RNA load in this phase is high in lymphoid tissues, but low in plasma [57].

After the peak of acute infection, concentrations of virus in blood are stabilized on individually different levels, the so-called set point, which is strongly associated with disease progression [14, 20, 50, 58]. During asymptomatic infection, the CD4 T cell count decreases continuously at an individually different but constant rate. A marked increase in the level of viral RNA in plasma is seen in advanced immunodeficiency when the CD4 T cell count has dropped to below 200/μl. This is usually interpreted as a final complete breakdown of the mechanisms that previously maintained a certain control of virus replication.

The concentration of p24 antigen in this chronic phase of infection largely follows that of the HIV RNA, but with two important differences. First, while all viral RNA in plasma is located inside viral particles, most p24 antigen is found outside. This is demonstrated by ultracentrifugation experiments in which very little of the total p24 antigen present in plasma could be pelleted (fig. 5a). In contrast, HIV RNA and reverse transcriptase could be quantitatively recovered from the ultracentrifugation pellet (not shown). The most obvious source for the extraviral p24 antigen are the numerous virions destroyed while sequestered in the lymphatics, but release from virus-producing cells or leakage from cells destroyed by viral or immune-mediated cytotoxicity is also possible (fig. 5b).

Another notable difference is seen in advanced disease when HIV RNA exhibits the above-mentioned marked increase (fig. 4). Cross-sectional studies have shown that there is no concomitant increase of p24 antigen. Instead, p24 antigen concentrations are similarly high in patients exhibiting 100, 200 or 350 CD4+ T cells/μl, while patients with 50/μl or below exhibit slightly lower antigen concentrations [41]. It is likely that the destruction of the follicular dendritic cell network, which is typically present in advanced HIV disease (bottom of fig. 4), leads to a decreased retention and destruction of particles, and more virus will reach the peripheral blood [59]. Thus, the apparent final rise of viral RNA in plasma (top of fig. 4) may rather be due to a gradually increasing redistribution of virus from the lymphatics to the bloodstream than represent a true increase in virus production. A reduced virus production in the final stage, as suggested by the decreasing concentrations of p24 antigen in plasma, would be in keeping with the total destruction of the CD4+ T cells.

Table 2. Diagnostic sensitivity of HIV-1 detection methods in pediatric samples [with permission, 40]

Age	Antigen neutralized	In-house PCR viral DNA	In-house PCR viral RNA	HIV-1 Monitor viral RNA
≤ 10 days	6/12 (50)	5/12 (42)	3/7 (43)	not done[a]
11 days to 3 months	10/10	8/8	7/7	6/6
>3 to 6 months	19/19	12/12	12/12	9/9
>6 months	191/191	66/66	26/26	120/120
>10 days	220/220 (100)	86/86 (100)	45/45 (100)	135/135 (100)

The number of positive/tested samples is shown with the percentage in parentheses.

[a] The sample positive in the antigen assay but negative by in-house PCR for viral DNA or RNA was also negative by the ultrasensitive HIV-1 Monitor version 1.5.

p24 Antigen and HIV RNA with Respect to Different Clinical Questions

Besides diagnosis of HIV infection which in Europe is increasingly done by means of combo tests that detect both antibody and antigen, virus component tests are needed for diagnosis of pediatric HIV infection, assessment of a patient's rate of disease progression, and control of ART (initial response to treatment, diagnosis of treatment failure). Studies addressing all these questions have been done. For all antigen assays the HIV-1 p24 Core Profile ELISA in combination with the ELAST® ELISA Amplification System (both available from Perkin Elmer Life Sciences) was used. Unless stated otherwise Roche's Amplicor HIV-1 Monitor® in versions 1.0 or 1.5 was used for quantification of viral RNA. For diagnostic purposes, qualitative in-house tests for viral DNA or RNA capable of detecting a single copy of HIV-1 DNA or cDNA were also used in early studies [32, 60].

Diagnosis of Pediatric HIV-1 Infection

A study conducted between 1994 and 1997 with prospective analysis of p24, HIV-1 DNA and RNA investigated the diagnostic sensitivity of p24 antigen and PCR-based tests in 232 samples from 61 HIV-1-infected untreated children born to HIV-positive mothers in Switzerland (table 2) [40]. All tests were 100% positive above 10 days of age. Below 10 days, p24 was confirmed positive in 6 of 12 samples. DNA PCR and in-house PCR for viral RNA both missed one of the samples positive for p24. When retested by the HIV-1 Monitor version 1.5 *ultrasensitive* assay with a detection limit of 50 copies/ml the sample was also negative. The diagnostic specificity of the p24 assay among 643 plasma samples from 246 uninfected children born to HIV-1-positive mothers was 99.2% after neutralization. Two (1.4%) of 141 samples tested with the in-house method for viral RNA were false-positive resulting in a diagnostic specificity of 98.6%. Thus, p24 was equal to RNA regarding diagnostic sensitivity and specificity in pediatric HIV-1 infection. The high sensitivity and practical utility of this procedure were also confirmed by others in children from Tanzania [61].

Diurnal Variation of HIV-1 p24 Antigen Concentration in Plasma and Precision

Few data are available on precision of the p24 antigen assay, but they suggest a higher precision than that of the HIV-1 Monitor assay. Diurnal variation of plasma HIV-1 load at four different time points each during two different days (a Friday and the following Monday) was studied in five HIV-1-infected children with implanted intravascular catheters after informed consent had been given. The investigations demonstrated that the p24 antigen levels had, with a mean log standard deviation (SD) that amounted to 0.057 (range 0.02–0.11), less variation than the HIV-1 RNA concentrations (mean log 0.108; range 0.07–0.15) [40]. In another study in which 8 different specimens were tested 3–4 times in an assay, the mean log SD of the antigen test was 0.07 compared to 0.11 for the Roche HIV-1 Monitor assay [44].

Prediction of Disease Progression

The predictive value of p24 antigen concentration was tested in two published studies. In a first, retrospective study involving 169 chronically infected adult Swiss patients with a median CD4+ T lymphocyte count of 140 cells/μl (range 0–1,500), p24 antigen and HIV-1 RNA concentrations were determined in a single sample collected in 1993–1994 and the predictive value of these markers regarding disease progression was compared.

Follow-up data included at least one further CD4+ T lymphocyte count and assessment of the clinical stage with a median observation period of 2.7 years (range 0.1–4.9). In CD4-adjusted Cox proportional hazard models, both RNA ($p < 0.005$) and p24 antigen ($p = 0.043$) were significant predictors of progression to AIDS. p24 was superior ($p = 0.032$) to RNA ($p = 0.19$; nonsignificant) in predicting survival. p24 was also a significant predictor of the CD4+ decline in 'CD4+-adjusted' models and was equivalent or superior to HIV-1 RNA depending on the group analyzed and the statistical test employed [41].

The prognostic value of p24 antigen was confirmed in a second study which involved first-visit plasma samples from 494 mostly black IVDU from Baltimore, Md, USA. This cohort had a median initial CD4+ lymphocyte count of 518/µl; 90 of the patients (18%) progressed to AIDS within 5 years. p24 antigen was strongly correlated with both HIV-1 RNA, as determined by bDNA assay ($r = 0.55$; $p < 0.0001$) and CD4+ lymphocytes ($r = –0.34$; $p < 0.0001$). p24 level >5 pg/ml predicted disease progression comparable to cutoffs of <350 CD4+ lymphocytes/mm^3 and >30,000 copies/ml HIV-1 RNA. Heat-denatured p24 antigen thus predicted subsequent clinical disease progression in early-stage HIV-1 infection, and was closely correlated with both CD4+ lymphocyte and HIV-1 RNA level [43].

ART Monitoring and Detection of Treatment Failures

The suitability of p24 antigen for ART monitoring was investigated in both adult and pediatric infection of patients in Switzerland. In a study of 23 adult patients with advanced disease who received a new, indinavir-containing treatment regimen, p24 antigen was detected as sensitively as viral RNA, namely in 75.6% of the samples (RNA 73.6%). Antigen and RNA levels in 79 samples positive for both markers correlated with $R = 0.714$ ($p < 0.0001$). This correlation was similar to that found in a different study in which HIV-1 RNA levels were determined in parallel by two different methods, namely the Amplicor HIV-1 Monitor and the NucliSens® HIV-1 RNA Quantitative Test [62]. Mean changes in levels of p24 antigen and RNA at eight time points correlated with $R = 0.982$ ($p < 0.0001$; fig. 6). In individual patients, the two parameters behaved similarly and in certain cases virtually identically [39]. Similar results were found in a prospective study of 25 children with a total of 230 analyzed samples in Switzerland. Here, the correlation of RNA and p24 antigen in individual samples was $R = 0.658$ ($p < 0.0001$). In most instances the treatment-induced changes were more pronounced for HIV-1 RNA than for p24. p24 levels showed significantly less variation than HIV-1 RNA [40]. Good correlation between HIV-1 RNA and p24 antigen ($R = 0.751$, $p < 0.0001$) was also observed with sequential samples from patients infected mostly with non-B subtypes [45].

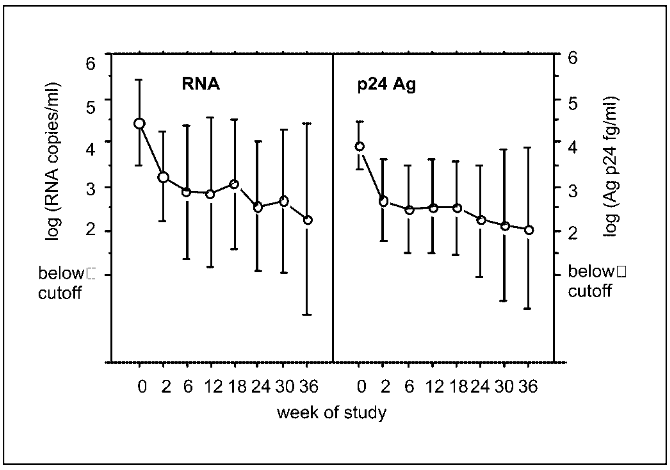

Fig. 6. Treatment-induced changes in concentrations of HIV-1 RNA and p24 antigen. Logarithmized mean values ± 1 standard deviation are shown [with permission, 39].

We also investigated 34 Swiss patients who were enrolled in 1997 into two treatment studies in which they were prospectively tested for viral RNA by the Roche HIV-1 Monitor version 1.0 and p24 antigen [63]. The data were evaluated regarding the response of these markers to ART and timely detection of treatment failures. We found that p24 antigen was detectable in 75.8% of 178 samples and HIV RNA in 73.9% of 138 samples. Correlation of the two markers was good ($R = 0.744$, $p < 0.0001$). Treatment failure, as defined by RNA concentrations, occurred in 14 patients (fig. 7). Secondary treatment failures with RNA rebounds from undetectable levels to less than 10^3 copies/ml in 2 patients with an undetectable viral load and 10^3 HIV RNA copies/ml, respectively, at baseline were not detected by p24 antigen. The two failures carried a low risk for secondary resistance mutations and were, as demonstrated by retesting with a still more sensitive p24 antigen assay, in principle detectable. The other 12 failures were detected on average 29 days earlier by p24 antigen than by RNA ($p = 0.020$), owing to slightly more frequent testing for p24 antigen than for RNA (2.7 vs. 2.4 tests until detection of treatment failure). Average costs of p24 antigen testing up to a failure were only 20.5% of those of RNA ($p < 0.0001$).

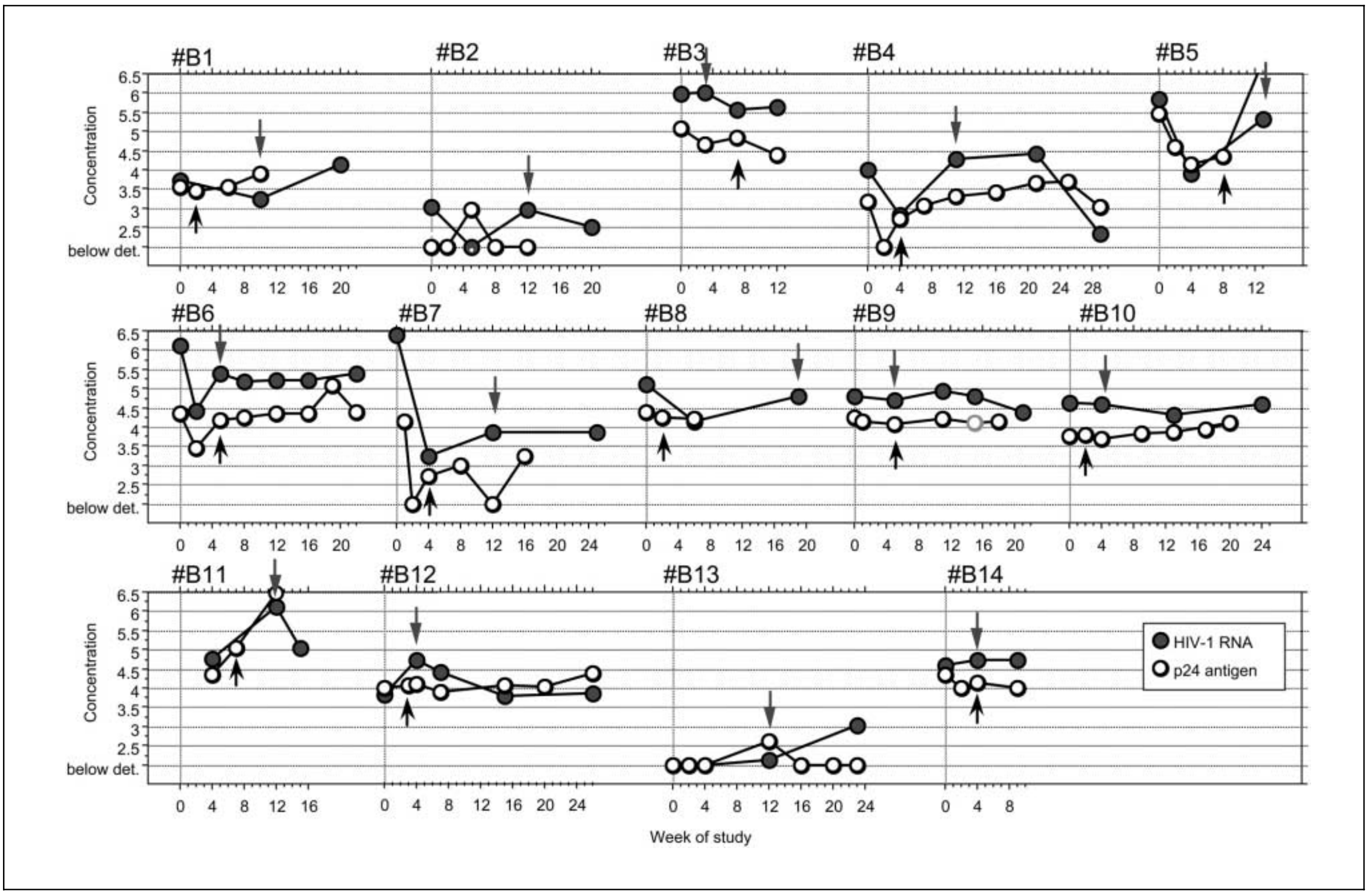

Fig. 7. Courses of HIV-1 RNA and p24 antigen in patients receiving ART and detection of treatment failure. Downfacing arrows mark the point of failure detection by PCR for viral RNA, upfacing arrows by ELISA for p24 antigen. Panels #B2 and #B13 do not contain upfacing arrows since the rebounds of p24 antigen in these patients were not confirmed by the subsequent measurement [with permission, 63].

These findings should not be interpreted to suggest that the p24 antigen test would be as sensitive as RT-PCR in detecting virus that rises only slowly after a long period of complete viral suppression. Complete suppression of replication will with time also deplete the stores of immune-complexed p24 antigen in the lymphatics. In the absence of the extraviral background of p24 antigen a certain concentration of virus particles in plasma is needed before p24 concentrations rise above the limit of detection, similar to the situation in acute infection (see below and fig. 8).

p24 Antigen in Patients with Stably Suppressed HIV RNA

The studies mentioned above demonstrate the use of the p24 antigen assay for monitoring of newly initiated treatments. We were also interested whether the assay would be useful in patients whose HIV RNA was stably suppressed by long-term ART. We therefore investigated p24 antigen concentrations prospectively in 55 patients whose viral RNA in plasma had previously been suppressed for at least 6 months under antiretroviral combination therapy. During a median follow-up of 504 days, CD4 counts increased by a median of 62 cells/year. By both univariate and multivariate linear regression analysis the level of p24 antigen, as expressed by the absorbance/cutoff ratio, was a significant inverse correlate of both the CD4 count in a sample ($p = 0.013$) and its annual change in a patient ($p < 0.0001$). p24 retained significance even among 48 individuals whose HIV-1 RNA, apart from occasional blips, remained below 400 copies/ml. Batchwise retesting of 70 samples from 5 such patients with a further improved procedure showed measurable p24 antigen in all but one sample and an inverse correla-

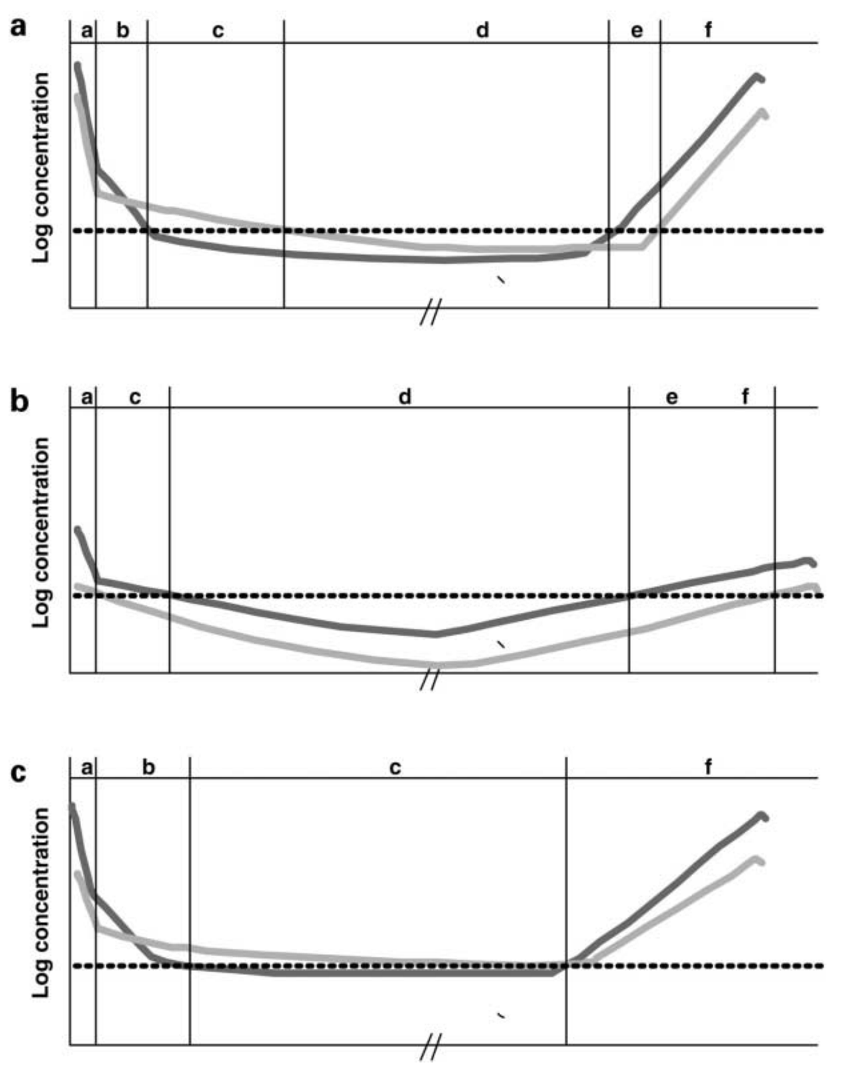

Fig. 8. Schematic representation of differential courses of HIV RNA and p24 antigen following initiation of ART leading to complete virus suppression and loss of treatment efficacy due to resistance mutation. Black line = HIV RNA; grey line = p24 antigen; dotted horizontal line = limit of detection. **a** Situation in which initial HIV RNA and, thus, particle-associated p24 is high compared to extraviral p24 antigen. The sequential phases a–f are: both HIV RNA and p24 antigen (largely particle-associated) decrease with same $t_{1/2}$ (a); both markers still detectable; they decrease with different $t_{1/2}$ (b); only p24 antigen detectable due to longer $t_{1/2}$ (c); both markers undetectable (d); viral RNA first detectable in viral failure because RT-PCR is more sensitive in detecting particles (extraviral stores of p24 antigen are depleted) (e); both markers positive (f). **b** Situation in which both initial HIV RNA and extraviral p24 are low: quick disappearance of p24 antigen (a); only HIV RNA detectable (c); both markers undetectable (d); slow increase of HIV RNA after low-grade resistance mutation leads to delayed increase of p24 antigen, due to depleted stores (e, f). **c** Virus suppression with HIV RNA concentration remaining just below the detection limit while p24 antigen, possibly fed by antigen released in the lymphatics, remains detectable at low concentration for a prolonged period of time [36].

tion with both the CD4 count ($p = 0.0331$) and percentage ($p < 0.0001$), thus confirming the prospectively generated data [36]. We have meanwhile also evaluated the relationship of p24 antigen concentrations to subsets of CD8+ T cells exhibiting the activation markers CD38 and/or HLA-DR in these patients and found highly significant positive correlations, even in a subgroup of samples in which HIV RNA was undetectable in a test with a detection limit of only 5 copies/ml. Thus, the concentration of p24 antigen is directly correlated with the number and percentage of those cells that represent the hyperactivation of the immune system, which is considered a key element of HIV pathogenesis [59]. These two studies demonstrate that HIV RNA measured by RT-PCR is not per se a more sensitive marker of HIV infection than is p24 antigen, even though it is admittedly more sensitive in detecting HIV particles [51].

Differential Course of HIV RNA and p24 Antigen during Changes in ART

Since the half-life of virus particles is short, initiation of efficient combination therapy in previously untreated patients leads to a rapid reduction of HIV RNA in plasma [10, 13]. The half-life of p24 antigen in the first phase of effective treatment is similar to that of HIV RNA, thus representing the decrease of particle-associated antigen

[63]. Similar to HIV RNA a second, slower decay phase was found which had a half-life of 42 ± 16 days. In contrast to HIV RNA, the antigen detected in this phase is not particle-associated, but consists of immune-complexed or free extraviral protein. As demonstrated by the two abovementioned studies and other, not yet published work such protein may still be present well after HIV RNA has become undetectable by the most sensitive assays. Based on the initial ratio of particle associated to extraviral p24 antigen, the efficacy of ART and the time point and severity of resistance mutations, different patterns of relationships between HIV RNA and p24 antigen during treatment follow-up can be found (fig. 8).

Detection of Viruses of Non-B Subtype

The results described above indicate that p24 is comparable to HIV-1 RNA when used for diagnosis of pediatric HIV-1 infection, as a marker of disease activity or progression or for treatment monitoring in Switzerland or the US where subtype B infections prevail.

Antiretroviral therapy is now becoming increasingly available to patients living with HIV/AIDS in many developing countries, due to significant reductions in antiretroviral drug prices. There is thus a rapidly increasing demand for inexpensive tests capable of assessing the need for antiretroviral therapy in a given patient and of monitoring the effect of such treatment. It is estimated that ART costs will be in the order of USD 300 per patient and year. The high costs of the above-described tests which currently amount to about USD 50–100 per test and the fact that at least two such tests, if not four, will have to be performed every year seem to preclude their use in the many less affluent countries and societies. In addition, these molecular-based tests require technically advanced and expensive facilities and equipment, as well as highly trained laboratory personnel. It is thus difficult to imagine that these tests could be sensibly used for monitoring of ART in developing countries. An inexpensive method for measuring the virus load could greatly improve the diagnosis and treatment of HIV infection worldwide.

With regard to a use of this test in developing countries, in particular Africa, it is important to assess the suitability for non-B subtypes. Only limited data regarding this issue are currently available. Lyamuya et al. [61] found a high diagnostic sensitivity in diagnosing pediatric HIV-1 infection in Dar es Salaam, Tanzania. Altogether, 123 of 125 samples from 76 PCR-positive infants were positive for p24 antigen (sensitivity = 98.7%). HIV-1 p24 antigen was found in all 18 samples collected at 1–8 weeks, in 35 of 36 samples collected at 9–26 weeks, in all 40 samples collected at 27–52 weeks, and in 30 of 31 samples collected 52 weeks after birth. The sensitivity of the assay was also assessed in a Swiss study of 103 individuals likely to be infected by non-B subtypes [64]. The tests assessed included three RNA-based assays including the Amplicor HIV-1 Monitor 1.5, the Quantiplex version 2.0 (bDNA), the NucliSens (NASBA), an ultrasensitive reverse transcriptase assay called PERT assay [65] and the improved p24 antigen assay. Subtyping was based on sequencing in the *env* gene. p24 was more sensitive than NucliSens or Quantiplex, but less sensitive than Amplicor or PERT assay. A more detailed, quantitative comparison showed that 2 samples with an HIV-1 RNA concentration above 10,000 copies/ml (one subtype A and one subtype C) were negative for p24 antigen. Other samples of these subtypes were, however, well recognized, even some in which HIV-1 RNA was not detectable or below the limit of quantification (400 copies/ml). In particular, the p24 antigen assay was also positive in one subtype O sample that was negative by all assays for HIV-1 RNA, but positive by the PERT assay. Good detection of subtypes A–F and circulating recombinant strains was also reported by others [44], and a group from Thailand recently reported good results with using an antigen kit of a different manufacturer in combination with Perkin-Elmer's tyramide signal amplification step [66]. These data suggest that the p24 antigen assay is not per se inferior to tests for HIV-1 RNA regarding recognition of different subtypes. However, this issue needs to be studied more extensively before the test is routinely used in non-B areas, and adjustments regarding the capture or tracer antibodies of the kit or development of an entirely new kit may prove necessary.

Sample Handling and Physical Stability of p24 Antigen

In an attempt to strengthen further the evidence for a predictive value of p24 antigen we recently conducted a study involving serum samples collected between 1989 and 1990 from 547 patients of all disease stages treated at the Zürich University. The study intended to directly compare the predictive values of HIV-1 p24 antigen and HIV-1 RNA. Unfortunately, HIV-1 RNA was found to be degraded in the majority of samples, and the study had to be restricted to the assessment of p24 antigen alone. Of the 547 samples, 92.5% had a p24 antigen concentration above the cutoff; these samples exhibited the same concentration distribution as previously noted in another study [41]. These data indicate that p24 antigen is much more stable than is viral RNA. In accordance with this there is no need for special 'plasma preparation tubes', expensive individual express delivery, and –70 °C freezers. Samples may be kept for several days at 4 °C before testing. Preliminary assessment of the effect of freezing-thawing cycles has indicated that one such cycle leads to about 3% loss of p24 antigen, with no further change after the third cycle (unpubl. data).

Costs

In the absence of a kit which contained all necessary ingredients for sample preparation, ELISA, and signal amplification it was difficult to assess the price of this test. Previous comparisons based on reimbursement by health insurances in Switzerland arrived at a price of CHF 50 (USD 30) for the antigen test and CHF 275 (USD 167) for a HIV-1 Monitor assay. A study performed in the US quoted USD 8 for the antigen assay and USD 75 for the HIV-1 Monitor assay including reagents and work [44]. Based on these data the costs of the p24 assay can be expected to amount to 10–20% of those of a HIV-1 RNA assay.

Conclusions

The different properties of p24 antigen and particle-associated HIV RNA require that the comparative clinical value of these markers is evaluated with reference to distinct fields of application including diagnosis of pediatric HIV infection, prediction of disease progression, and monitoring of ART. A simple assessment of the sensitivity of antigen testing using samples previously found positive for HIV RNA (which is taken as the gold standard) will not reveal that p24 antigen, even though it is less sensitive in acute HIV infection, is as good a predictor of disease progression as is viral RNA. Such superficial evaluations will also miss the point that p24 antigen correlates significantly with immune parameters considered crucial to HIV disease in patients in whom HIV RNA is no longer detectable, since samples with undetectable HIV RNA would be considered a priori to contain no HIV. In contrast, the studies reviewed here indicate that p24 antigen is as relevant to the biology of HIV disease as is viral RNA and that it is comparable to the latter with regard to sensitivity and specificity, prediction of progression to AIDS or death, and useful for monitoring of ART. Unlike HIV-1 RNA measurement, this simple, considerably less expensive and easily automatable procedure does not require cumbersome sample transport and pretreatment procedures. Further studies on p24 antigen are highly warranted; in particular, they should aim at validating the test for non-B subtypes.

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