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MINIREVIEW

Use of Virologic Assays for Detection of Human Immunodeficiency Virus in Clinical Trials: Recommendations of the AIDS Clinical Trials Group Virology Committee

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INTRODUCTION

The AIDS Clinical Trials Group (ACTG) offers a unique opportunity for studying the clinical efficacy of therapies targeted at human immunodeficiency virus (HIV) and AIDS-associated opportunistic infections in a cross-section of medical institutions spread throughout the United States. Early drug trials relied heavily on clinical endpoints as the measure of drug efficacy. As patients live longer and healthier lives, these endpoints become less useful. The need to obtain a more immediate answer to the question of drug efficacy has placed an increasing emphasis on monitoring the antiviral activity of these compounds in vivo.

The following is a review of the application of virologic assays used to detect HIV type 1 (HIV-1) within the context of multicenter clinical trials conducted by the ACTG. It represents the collective effort at standardizing procedures in over 50 U.S. virology laboratories that conduct clinical trials sponsored by the Division of AIDS, National Institute of Allergy and Infectious Diseases. The assays utilized are, for the most part, based on consensus protocols that were developed by the ACTG virologists and are available in the ACTG Virology Laboratory Manual. The review includes a brief description of these assays, their specimen and testing requirements, and their indicated use in areas such as diagnosis and monitoring of drug efficacy. For a more comprehensive discussion of the assays themselves, the reader is referred to an earlier review by Jackson and Balfour (20).

ANTIGEN DETECTION

Standard p24 assay. (i) Brief description. The HIV p24 enzyme immunoassay (EIA) utilizes an antigen-capture procedure to measure the presence or absence of the major viral core protein. This protein may circulate freely in the plasma or be bound by circulating p24 antibody. Free p24 antigen may be detected during primary infection, before specific antibody is formed, or it may reemerge later in the course of HIV infection as a reflection of the progressive increase in viral load and/or the loss of p24 antibody, both of which have been described as correlates of advanced disease (47). The lack of detection of p24 antigen during the often extended period of asymptomatic infection reflects the lower viral burdens seen during this period or its binding by p24 specific antibody. The latter is the basis for the development of the immune complex-dissociated (ICD) p24 antigen assay described below, which has sought to increase the prevalence of this marker in infected persons. Even with the latter procedure, however, p24 antigen has not been found to be a universal viral marker, and this remains one of its limitations in diagnostic situations (e.g., neonatal infection) or as a parameter to follow in therapeutic trials.

Numerous commercial p24 antigen detection kits are available, and each utilizes a colorimetric endpoint to measure the amount of antigen present. By using a standard curve, optical densities obtained from the assay are converted into a concentration of the antigen, expressed as picograms per milliliter. Three such kits are used by ACTG laboratories: Abbott, Coulter, and Dupont. In order to make results from the different manufacturers comparable, each laboratory performing the assay uses, in addition to the manufacturer's standard, a set of standards and controls provided by ACTG's Quality Assurance Program. A reference laboratory provides each participating site with a reference standard of purified p24 antigen at a concentration of 400 pg/ml. The antigen is an affinity-purified preparation that is derived from the IIIB strain of HIV-1 and is diluted in phosphate-buffered saline containing 1% bovine serum albumin and 0.5% Triton X-100 (E. I. du Pont de Nemours, Inc.). Each laboratory then dilutes this standard in a manner specific to the EIA kit employed. For example, the standard concentrations employed for the Abbott kit are 200, 100, 50, and 25 pg/ml, and for Coulter and Dupont kits, the concentrations of the standards are 100, 50, 25, and 12.5 pg/ml. Reference standards are run in duplicate in each assay.

Some variability does exist between individual kits, and this may reflect differences in the capture antibody (which is polyclonal in the case of Abbott and monoclonal in the case of Coulter and Dupont) or differences in the matrix to which the capture antibody is bound (e.g., polystyrene beads versus microtiter wells). The Quality Assurance Program mandates the use of quality control check samples in each assay in order to normalize results obtained with the different manufacturers' kits. The check samples contain 100, 25, and 0 pg of purified p24 antigen (the same material used in

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TABLE 1. ACTG laboratory agreement on p24 antigen values over time

Date tested	Acceptable coefficient of variation (%)	Laboratory agreement (%)	Acceptable entry criterion for clinical trials (pg of p24/ml)
03/88	10	53	70
05/88[a]	10	73	70
05/88	10	81	70
12/89	10	74	70
03/90	10	89	70
05/90	10	84	70
08/90	10	91	70
12/90	10	91	70
02/91	5	79	70
05/91	5	86	70
09/91	5	87	70
01/92	5	87	25
05/92	5	91	25

[a] Uniform reference standards and controls were introduced at this time.

the calibration reference standards) per ml, which has been placed into plasma or medium (depending upon whether one is assaying patient serum or plasma samples or culture supernatants).

All ACTG labs routinely undergo periodic proficiency testing to ensure that they can accurately perform the assay. Table 1 documents the experience with this assay, using three manufacturers' kits, within the ACTG. Prior to the introduction of standardized reagents in May 1988, only half the laboratories were in ±10% agreement on any one p24 antigen value obtained from testing a panel of coded patient specimens. Coded panels of 21 specimens containing HIV p24 antigen in the range of 0 to 160 pg/ml, in triplicate, were randomized and analyzed by the laboratories on a periodic basis. These panels were designed to measure both inter- and intralaboratory reproducibility. By the end of 1990, 90% of the laboratories were in accord. In 1991, the acceptable coefficient of variation on the assay was changed to within 5%. By mid 1992, over 90% of the laboratories were in ±5% agreement.

(ii) Specimen requirements. The assay uses serum or plasma (250 μl), though serum is most commonly used. Serum and plasma samples should be stored at −70°C after they are obtained. The assay may be run in real time, or samples may be frozen back for batch testing at a later date.

(iii) Testing requirements. Real-time testing is performed for entry (inclusion) criteria on protocols and for determination of baseline values. Two specimens obtained at least 72 h apart in the month prior to entry are recommended. At the time of study, when the interval for acquisition of sera is monthly or longer, specimens are usually run in a batched fashion from frozen material with baseline sample(s) included. Subsequent batch testing should include a certain percentage of the previous batch assayed run in tandem to rule out problems with assay methodology or specimen degradation. In some protocols, testing is performed weekly, or monthly, on a real-time basis. For post-therapy follow-up, confirmatory batch testing may be advisable at the conclusion of the study.

(iv) Indicated use: entry criteria. The utility of the assay is limited by the ability to detect p24 antigen (Table 2). In patients with $CD4^+$ counts between 200 and 500/mm^3, the percentage of patients positive for p24 is approximately 20% (17). Patients with $CD4^+$ counts under 200/mm^3 and/or who have been diagnosed as having AIDS or AIDS-related complex are more likely to be positive for p24 (positivity rates range from 37 to 95%) (7, 51). For patients who have demonstrated p24 antigenemia, a decrease in the levels of p24 in plasma is a useful means to measure antiviral efficacy. Many ACTG clinical trials, especially those involving the early evaluation of drug efficacy, have demonstrable p24 levels as an entry criterion. The high level of reproducibility and lack of interlaboratory variability, as demonstrated by performance on the proficiency panels (Table 1), have resulted in an increased confidence of the results obtained from the assay at the lower limits of sensitivity. Thus, the recommended p24 value for entry into trials has been changed from 70 pg/ml (when a 10% coefficient of variation was acceptable) to ≥25 pg/ml with the presently acceptable 5% coefficient of variation among laboratories. A 50% or

TABLE 2. Characteristics of HIV-1 virologic assays applicable to clinical trials

Assay	% of positive patients	$CD4^+$ range (cells/mm^3)	Viral parameter measured	Comments
p24 antigen	20 37–95	200–500 <200	Free viral antigen in serum or plasma	When present, useful as an easily measurable marker whose decline indicates active in vivo antiviral activity, helpful in the early evaluation of new agents.
ICD p24	45–70 75–100	200–500 <200	Immune-complexed viral antigen in serum or plasma	Acid treatment of serum or plasma results, in general, in heightened detection of p24 antigen as a result of its release from immune complexes. Useful in neonatal diagnosis of HIV-1 infection and in increasing the prevalence of this marker in potential study populations.
Plasma viremia	75–100	<200	Infectious cell-free virus	Mainly useful in more advanced disease populations given low prevalence in patients with $CD4^+$ counts >200/mm^3. When present, however, a useful quantitative marker to monitor response to antiretroviral therapy.
PBMC culture	95–100	<500	Infectious cell-associated and amplifiable virus	Early experience suggests this is a useful marker to monitor changes in viral titer on therapy. Expensive and labor-intensive, however.
DNA PCR	100	<1,000	Cell-associated proviral DNA	Useful in documenting neonatal HIV-1 infection and in assessing viral burden within the host.
RNA PCR	100	<1,000	Cell-free and/or cell-associated viral RNA	Techniques are still in development but promising as a marker of active in vivo virus expression which may be applicable to patients at all disease stages. Responses to antiretroviral and immune-based therapies are currently under active investigation.

greater fall in antigen concentration on therapy can be reliably detected.

(v) **Diagnosis.** The serum p24 antigen assay appears to be less sensitive than either culture or polymerase chain reaction (PCR) in detecting HIV-infected children at birth. During the first two months of life, serum antigenemia can be detected in only 50% of infected infants, but by 6 months, close to 90% of samples are positive (3). In primary infection of adults, there is an abrupt rise in p24 antigen production, which subsequently declines with the advent of p24 antibody production (10, 47). Within the context of ACTG protocols, the cutoff for a positive sample is determined by adding 0.05 to the mean optical density of the negative controls. This has been chosen because it generally falls at least 4 standard deviations above the mean of the negative controls and thereby provides statistical assurance that any sample with an absorbance value above this cutoff will be a true positive result. The Quality Assurance Program employs this cutoff rather than the manufacturer's cutoff because it provides standardization across assays and because internal testing and statistical analysis have confirmed its reliability when the recommended reference standards are used. It also permits accurate detection of antigen concentrations in serum or plasma down to the level of approximately 9 pg/ml.

(vi) **Monitoring therapy.** In 1988, Chaisson et al. successfully demonstrated a decline in p24 antigen levels in patients undergoing antiretroviral therapy which was not seen in placebo controls. This decline in p24 antigenemia was associated with a corresponding increase in the number of $CD4^+$ cells (7). Subsequent ACTG studies have demonstrated similar results (14). Moreover, drugs having little or no antiretroviral effect in vivo do not result in loss of antigen (51). Most recently, measurements of decline in p24 antigen levels have been found to correlate with a corresponding reduction in rate of progression to clinical endpoints (26, 55). Patient variability in this assay may be quite high; thus, a sustained 50% drop in concentration, based on two sequential measurements obtained over time, is considered a positive response to therapy. A nonsustained fall in p24 antigen may indicate lack, or loss, of therapeutic effect. The latter may be an indication of the emergence of resistant strains or of a suboptimal drug concentration. At the present time, the p24 antigen assay is one of the better validated markers of antiretroviral efficacy, and its use in phase I and II trials is recommended. This assay has limited application in trials targeted at groups with higher $CD4^+$ counts, such as immune-based therapy trials.

ICD p24 Assay. (i) **Brief description.** The HIV ICD p24 antigen assay is a modification of the standard p24 antigen assay which seeks to detect circulating p24 antigen that is bound by native p24 antibody and thus "hidden" from detection by the standard enzyme-linked immunosorbent assay. This is accomplished by disrupting immune complexes by the simple technique of acid treating serum, or plasma, prior to the performance of the standard p24 antigen assay (25, 29, 41). Specific p24 antigen and antibody complexes are formed early in the course of primary infection, and detection of these complexes increases during the progression of the disease (2, 37, 41). Sera that contain detectable p24 antigen in the standard assay frequently yield higher values after acid treatment. One cautionary note: sera that contain only low levels of uncomplexed p24 antigen may convert to negative, following acid treatment, because of degradation of free p24 antigen by the procedure. Each of the three major commercial suppliers of p24 antigen kits (Abbott, Coulter, and Dupont) has developed ICD p24 procedures and can distribute the appropriate reagents. In brief, the ICD procedure involves incubating an aliquot (e.g., 100 μl) of serum or plasma with an equal volume of an acid glycine reagent (pH 1.7 to 2.0). The temperature and time of incubation vary from 37 to 70°C and from 10 to 90 min, respectively, depending upon the kit employed. The specimen is then neutralized with Tris buffer, and the standard procedure for the EIA is carried out. Reference standards and controls are under development by the Quality Assurance Program. At the present time it is recommended that, when data from multiple sites are necessary, the assay be limited to use of one manufacturer's kit and one set of standards and controls. The commercial standards should be included in each assay along with a manufacturer-supplied positive control (a sample that will predictably yield a higher value after acid treatment). Results for specimens that convert from negative to positive following acid treatment or that are positive at the lower limit of detection of this assay (6 to 12 pg/ml) should be confirmed by specific neutralization with p24 antibody. Confirmation is of critical importance when applying this assay to neonatal or perinatal diagnosis.

(ii) **Specimen requirements.** Sera or plasma can be used for this assay, and 100 to 200 μl is generally recommended; the assay can be adapted to volumes as low as 75 μl. There is a threefold dilution involved in the first step of the assay, which is corrected for in the final calculation of picograms per milliliter. Sera or plasma may be assayed in real time or from frozen material that has been stored at −70°C.

(iii) **Testing requirements.** Testing requirements are similar to those for the standard p24 assay. They involve real-time testing (for protocol inclusion criteria and determination of baseline values) or frozen batch testing (for specimens obtained on study). As with the standard p24 assay, two specimens obtained at least 72 h apart in the month prior to entry are generally recommended, as is the inclusion of baseline sample(s) with batch testing of frozen study specimens.

(iv) **Indicated use: entry criteria.** One of the major indications for employing the ICD p24 assay is to increase the prevalence of this quantifiable virologic marker in a study population. This may prove particularly valuable in populations with earlier disease, in which the frequency of detectable p24 by the standard assay is lower than in patients with more advanced disease. Data from a number of laboratories suggest that this assay can routinely increase the prevalence of detectable p24 antigen in a population (2, 41). For example, seroprevalence can increase from approximately 40 to 50% to 75 to 100% in adult patients with $CD4^+$ cell counts $<200/mm^3$ and from 17 to 25% to 45 to 70% in patients with $CD4^+$ counts between 200 and $500/mm^3$ (18a, 36, 52). Similar trends have been reported in neonatal populations (43). As an entry criterion for protocols, an ICD p24 value of at least 25 pg/ml is generally recommended.

(v) **Diagnosis.** Another major indication for employing the ICD p24 assay is to improve the speed and reliability with which neonatal infection is diagnosed (38). In perinatally infected children, HIV-1 p24 antigen is frequently bound to antibodies and is undetectable. Acid treatment results in a 69% increase in antigen-positive sera (43). While no false positives were detected in this study, others have observed that borderline results can give rise to false positives (4, 8, 35, 45). It is recommended, therefore, that a specific confirmatory neutralization assay be performed when sera yield low-positive values or conversions from negative to positive occur in comparison to the standard p24 antigen assay. Currently, cutoff values are determined for each commercial

adaptation of the acid dissociation technique by the particular manufacturer.

(vi) Monitoring therapy. Bollinger et al. have demonstrated that the ICD p24 assay was fivefold more sensitive than the standard p24 antigen detection assay in asymptomatic patients and that treatment of those patients with zidovudine (ZDV) resulted in a sustained 50% decrease in ICD p24 antigen from the baseline level, compared with an increase in p24 antigenemia in placebo-treated controls (2). Similar observations have been made for patients receiving dideoxyinosine (44). More recently, experience with this assay suggests that in individuals who are given a nonnucleoside reverse transcriptase inhibitor, the level of ICD p24 declines serially, in a fashion similar to that seen with the standard p24 antigen test, and that it can rise in association with the emergence of antiviral resistance to the drug (15, 46). The guidelines for entry criteria and positivity used in these studies for ICD p24 are the same as for the standard p24 assay. A sustained 50% fall in ICD p24 antigen concentration is considered a positive response to therapy. A nonsustained fall in ICD p24 may indicate an ineffective antiretroviral agent or loss of antiviral effectiveness.

VIRAL ISOLATION

PBMC culture. (i) Brief description. The quantitative peripheral blood mononuclear cell (PBMC) assay measures the number of patient cells in the peripheral blood necessary to produce infectious HIV in culture—the greater the number of cells necessary to produce a positive result, the lower the virus load in the PBMC. Results are usually expressed as infectious units per 10^6 cells. Each sample of patient cells is cultured with phytohemagglutinin (PHA)- and interleukin-2-stimulated HIV-positive donor PBMC for 14 days. The assay is performed in duplicate in a 24-well tissue culture plate with six fivefold dilutions, beginning with 10^6 patient PBMC. Supernatant from each well is assayed by the standard p24 EIA method to determine positivity and level of production of viral protein. All ACTG laboratories currently participate in a Quality Assurance Program to ensure that the assay is accurately performed and that the test results are comparable from laboratory to laboratory.

A qualitative PBMC macroculture assay may be useful in instances where increased sensitivity is desired, as in diagnosis of infection, and where greater volumes of high-titer virus are necessary for subsequent viral resistance assays. A standardized qualitative macroculture assay has been utilized by ACTG since 1989, and there is a 95% concordance in results between laboratories (19). The quantitative microculture assay can also be used for a qualitative endpoint, as in the case of diagnosis of infection.

(ii) Specimen requirements. The assay utilizes heparinized or citrated peripheral blood (20 ml), from which PBMC are isolated by a Ficoll-Hypaque gradient (19, 21). Blood must be processed within 24 h, and cells not used in the assay are to be frozen in liquid nitrogen.

(iii) Testing requirements. Real-time testing is performed at baseline and at specified time intervals thereafter. Phase I and II studies for dose range, safety, and preliminary efficacy of an antiretroviral agent will have shorter time intervals (weekly to monthly). Phase III randomized clinical efficacy studies, comparing the accepted standard of care with the new therapy, will collect specimens monthly to quarterly. Quantitative cultures should be set up within 24 h of blood collection and immediately after Ficoll Hypaque separation.

Currently, all assays are performed in real time. The assay works equally well on frozen cells; however, some lowering of titer may occur (16).

(iv) Indicated use: diagnosis. For a specimen to be considered positive by either the quantitative microculture assay or the qualitative macroculture assay, the sample supernatant must contain 30 pg or more of p24 antigen per ml, as determined by the standard p24 EIA. This arbitrary value has been defined historically on the basis of average positive values from the various kit manufacturers and is unrelated to that used for determining positivity in sera or plasma.

(v) Monitoring therapy. HIV can be cultured from the peripheral blood of virtually all HIV-seropositive patients (6, 11, 18, 21, 22, 54). However, the number of infected cells in the peripheral blood can vary. Ho et al. observed that mean titers were significantly higher in patients with AIDS and AIDS-related complex than in asymptomatic patients; 2,200 and 2,700 versus 20 infectious units/10^6, respectively (18). The applicability of this assay for monitoring changes in cellular virus load concurrent with antiretroviral therapy is presently under investigation. The titer (number of patient cells containing at least one infectious unit per 10^6) is determined by the method of Spearman-Karber (34) and is reported as infectious units per 10^6 cells. The inherent assay variation is 1.2 $\log_{10}$ (11a). Thus, a reduction in titer by greater than 1 log which is sustained over two samples is considered a positive therapeutic result. The efficacy of this assay in a multicenter clinical trial has been demonstrated in ACTG protocol 143, where patients experienced a greater than twofold decrease in viral titer from baseline while on study drug (43a). The assay is presently being utilized in all phase I and II studies and in subsets of patients in phase II and III trials. It may also prove to be useful in immune-based therapy trials where $CD4^+$ levels are in the 200 to 500-mm^3 range.

Plasma culture. (i) Brief description. The quantitative plasma culture assay measures the amount of cell-free infectious HIV in patient plasma—a measure of plasma viremia. The assay is similar in design to that used for quantitative PBMC culture. Each sample of patient plasma is cultured with stimulated HIV-negative donor PBMC for 14 days. The assay is performed in duplicate in a 24-well tissue culture plate with 6 fivefold dilutions of plasma. Supernatant from each individual well is assayed by the standard p24 EIA method to determine positivity and level of produced viral protein.

(ii) Specimen requirements. The assay utilizes plasma from citrate heparinized peripheral blood (10 ml). For best results, blood should be processed within 4 to 6 h of draw. Plasma not assayed immediately should be stored at −70°C for future use.

(iii) Testing requirements. Real-time testing is performed at study baseline. This is usually a qualitative culture with undiluted plasma (2 ml with 10^6 donor PBMCs) to determine whether the patient is plasma viremic. Subsequent plasma samples from patients with positive baseline cultures are tested quantitatively by real-time assay or are batch tested within 3 months along with previously frozen baseline samples. As with the quantitative PBMC culture, phase I and II studies will have shorter time intervals (weekly to monthly) than phase III studies (monthly to quarterly).

(iv) Indicated use: monitoring therapy. Plasma viremia is not apparent in all HIV-positive patients. Infectious virus is rarely detectable in patients with $CD4^+$ cell counts above 500/mm^3 (11, 28, 47, 54). As the $CD4^+$ cell count drops, the percentage of patients with detectable plasma viremia will increase. For practical purposes, plasma viremia cultures should only be attempted when $CD4^+$ counts are <200/mm^3

and are accompanied by a positive baseline cell culture. Any sample well containing 30 pg or more of p24 antigen per ml (as determined by the standard p24 EIA) is considered positive. The titer (highest dilution of patient plasma containing at least one infectious unit) is determined by the Spearman-Karber method (34) and is reported as infectious units per milliliter. As with the quantitative PBMC culture, a greater than 1 log decrease in titer, an indication of reduced viral load in plasma, which is sustained over two samples, is considered a positive therapeutic result. Various clinical trials have shown that patients on zidovudine therapy exhibit one- to twofold log decreases in plasma viremia titers (18, 28, 47). ACTG studies are continuing to examine the efficacy of measuring plasma viremia.

PCR

DNA PCR assay. (i) Brief description. The qualitative HIV-1 DNA PCR detection assay has been used primarily to detect the presence of HIV-1 DNA sequences in peripheral blood cells (32). Two commercial HIV-1 DNA PCR assays have been validated for use in ACTG protocols: the Roche assay, which uses biotinylated primers (SK 462-431) to amplify and detect a highly conserved HIV-1 *gag* sequence (23, 56), and the Perkin-Elmer/GenProbe assay, which uses SK 38-39 primers to amplify a highly conserved portion of the *gag* region, which is then detected with a chemiluminescent probe (42, 56). There are many technical problems associated with PCR; thus, all ACTG centers performing the assay must participate in the Quality Assurance Program (12, 31). All ACTG labs performing the assay use quantitative HIV-1 DNA copy standards, provided by the Quality Assurance Program, to establish the sensitivity of each assay run. In addition, coded cell pellets are tested on each assay run performed to monitor sensitivity and specificity in real time. To be valid, a run must have no false positives or false-negative controls. Laboratories must also undergo periodic proficiency testing with patient whole-blood specimens to ensure that they can accurately perform the assay.

A quantitative HIV-1 DNA PCR assay is not recommended at this time for use in ACTG protocols. Although quantitative PCR measurements have been made by a limiting-dilution approach (50) or in comparison with external (13, 49) and, more recently, internal (24) controls, there is no standardized assay that is currently available and has been validated. In addition, while the quantitative methods have shown an association of increased viral load with disease progression (58) and increases in other markers of viral load (22, 47, 50), interpatient variability is great (24). Thus, the assays are not thought to be widely applicable at this time for use in ACTG protocols. However, in anticipation of better therapies and the development of accurate quantitative HIV-1 DNA PCR assays, washed cell pellets consisting of 10^6 PBMC after Ficoll-Hypaque separation of whole blood (1 ml) can be stored for future testing. Cell pellets or extracted cell lysates should be frozen at −70°C until tested.

(ii) Specimen requirements. A minimum volume (100 μl) of whole blood is collected in EDTA or citrate. Alternatively, a washed cell pellet consisting of 10^6 PBMC after Ficoll-Hypaque separation of whole blood (1 ml) can be stored for future testing. Cell pellets or extracted cell lysates should be frozen at −70°C until assayed.

(iii) Testing requirements. Real-time testing is performed for HIV-1 diagnosis and protocol entry criteria. Because of the technical problems associated with the assay and the desire to eliminate all false positives, it is recommended for HIV-1 diagnosis that cell lysates be amplified in duplicate, and detected singly, on two separate specimens.

(iv) Indicated use: diagnosis and entry criteria. Initial reports suggested that HIV-1 DNA PCR could detect HIV-1 in antibody-negative persons prior to seroconversion (57). Most reports have not confirmed these earlier findings yet did demonstrate a specific correlation with antibody and culture positivity in association with HIV-1 infection (5, 22). In the few cases where persons are PCR positive, but seronegative, they have also been p24 antigen positive, i.e., in the acute phase of illness (5, 24, 47). Thus, HIV-1 DNA PCR is felt to be a very specific and sensitive assay for use in diagnosing infection. This is particularly important for the early diagnosis of infection in infants. Borkowsky et al. have shown that the PCR assay was comparable to cocultivation for detecting infection (3). Since the turnaround time for PCR is significantly shorter than that of culture, the assay may be critical in determining early entry of infants into clinical trials. For a specimen to be considered positive by HIV-1 DNA PCR, the absorbance of both amplifications must be greater than 0.35 optical density units for the Roche assay or greater than 10,000 relative light units for the Gen Probe assay. For the diagnosis of HIV-1 infection, two specimens collected at different times must be HIV-1 positive by PCR as defined above. There is still some question at this time regarding confirmation of the PCR positive results by culture.

(v) Monitoring therapy. At the present time, neither the quantitative nor qualitative assay appears to be helpful in assessing the likelihood of a clinical response to experimental therapies, as preliminary data indicate that proviral load does not change significantly, at least in nucleoside experienced patients receiving mono- or combination therapy with nucleoside analogs (40).

Plasma RNA PCR. (i) Brief description. Currently, there is no standardized quantitative HIV-1 RNA assay available or validated. Moreover, issues regarding the biological variability, the variability of the reverse transcription step, the effect of processing and storage time on deterioration of genomic RNA, and the effect of different anticoagulants on PCR have not been resolved. However, such an assay is likely to be helpful in assessing the likelihood of a clinical response to experimental therapies. Aoki-sei et al. and Yerly et al. have shown that levels of circulating genomic HIV RNA correlate with p24 antigenemia and decreasing $CD4^+$ counts (1, 58). The former group observed in a preliminary study with didanosine that there was an apparent decrease in HIV RNA during therapy (1). Similar findings indicating that levels of plasma viremia change significantly, at least short term, in response to certain drug therapies have been observed by others (19a). A pilot study is presently being undertaken by a number of ACTG laboratories to assess the variability of the various genomic HIV RNA assays, which will subsequently lead to a larger multicenter study to validate the assay in a clinical protocol. However, no specific quantitative plasma HIV-1 RNA PCR assay is recommended at this time for use in ACTG protocols.

(ii) Specimen requirements. In anticipation of the development of accurate quantitative HIV-1 RNA PCR assays, 1.5 ml of citrated plasma may be frozen and stored at −70°C within 6 h of collection for future testing.

IN VITRO DRUG SUSCEPTIBILITY OR RESISTANCE

Phenotypic PBMC-based assay. (i) Brief description. The in vitro susceptibility assay measures the degree of drug inhibition of HIV p24 antigen production in PBMCs following

acute infection with a viral isolate. A two-step procedure that includes an initial determination of the infectivity titer of a clinical isolate followed by a drug sensitivity assay is used. This consensus assay (23a) utilizes PBMCs in order to maximize the number of clinical isolates which can be analyzed. Virus can be isolated in 80 to 95% of individuals with $CD4^+$ counts less than 500/mm^3 (11, 18, 21). This is in comparison to the 30 to 50% isolation rate obtainable by using MT2 cells (33). Both the isolation and the susceptibility steps are performed in a microtiter system using 96-well plates. Infectivity titer determination of clinical isolates utilizes a streamlined endpoint dilution and is analyzed by the Spearman-Karber statistical method (34). After the titer of a virus stock is determined, an aliquot containing 1,000 50% tissue culture infectious doses per 10^6 PBMC is used as an inoculum in each of a second set of in vitro infections of PHA-stimulated PBMCs (the sensitivity assay). Infected wells, in the absence of drug and at each of a number of drug concentrations, are fed with a 50% medium exchange/cell split at 4 days, and supernatant fluid is harvested after 7 days of culture. HIV p24 antigen is quantitated, and the 50% inhibitory concentration (IC_{50}) of drug is determined by using the median effect equation (9).

(ii) Specimen requirements. A cell-free culture supernatant fluid derived from PBMC coculture of either patient PBMC or plasma with a minimum titer of 2,000 50% tissue culture infections doses per ml is required for the assay. This is referred to as an isolate or a virus stock. The stocks may be derived from a qualitative macroculture, qualitative microculture, or quantitative microculture. Virus isolates obtained from microcultures are likely to require some expansion, in order to reach adequate levels of infectivity for the sensitivity assay. A standardized method for specimen collection or stock expansion for the sensitivity assay, using what are called "ministocks" generated from quantitative microcultures, has recently been agreed upon: fresh growth medium, containing PHA-stimulated donor cells, is added to each of the duplicate wells containing the highest concentration of HIV-infected PBMCs. Virus is allowed to grow for an additional 3 to 4 days. The amplified stock is then aliquoted and frozen at −70°C. The same approach can be used to prepare a specimen for infectivity titration and for susceptibility testing from qualitative cultures.

(iii) Testing requirements. It is optimal to test in parallel serial isolates from an individual patient prior to starting therapy and on (or after) therapy in order to best assess development of resistance to the therapeutic agent. Cutoff values for sensitivity and resistance have not yet been established for this assay. However, in the case of ZDV, an isolate that has an IC_{50} that is 100-fold greater than the IC_{50} of a previously susceptible isolate from the same patient can be classified as resistant.

(iv) Indicated use: epidemiologic studies. Reduced drug susceptibility for HIV-1 was first observed in 1989 by Larder et al. (33). There have been many subsequent reports regarding the emergence of drug resistance; however, the association of resistant phenotype with clinical failure has yet to be determined. Recently, Tudor-Williams et al. have shown that children who were clinically unresponsive to monotherapy with ZDV had reduced ZDV susceptibility (53). Analyses of isolates from adult clinical trials are now under way to ascertain whether isolation of an in vitro drug-resistant HIV phenotype is causally associated with clinical failure.

(v) Entry criteria. Currently the assay is not used as an entry criterion on any protocols. However, the assay may be valuable in small phase I and II studies to retrospectively evaluate the degree of ZDV resistance at entry for stratification analysis. The assay has approximately a 2- to 4-week turnaround time after an isolate is obtained.

(vi) Monitoring therapy. A temporal correlation between emergence of resistant virus and an increase in viral load, as measured by increases in circulating p24 antigen and plasma viremia, has only been suggested to date for nonnucleoside reverse transcriptase inhibitors (27, 46, 48) and more recently ZDV (30, 39). On the basis of these data, however, it is recommended that the assay be used in phase I trials of new drugs to monitor the emergence of drug resistance. The emergence of drug resistance cannot yet be used as an indication of loss of clinical effectiveness, though resistance to antiviral therapy is likely to be a factor in the decline of therapeutic effectiveness. The greatest experience with the phenotypic resistance assay described above is with ZDV, but any reverse transcriptase inhibitor (dideoxyinosine, dideoycytidine, nonnucleoside reverse transcriptase inhibitors) can be tested. Although there is less evidence from studies of agents with different action mechanisms, the assay should be broadly applicable to all agents; confirmation of applicability to new agents by pilot studies is recommended.

CONCLUSIONS

Each of the assays described above has different characteristics which make it more, or less, applicable in clinical trials. The selection of which assay to use will depend on the patient population being studied and the objectives of the trial. Table 2 summarizes the characteristics of these assays with respect to their expected prevalence in an untreated patient population based on $CD4^+$ count. Assays with similar sensitivities may measure quite different aspects of viral load and thus are likely to have greater or lesser utility, depending upon the antiviral compound in question. In many cases, specific validation of a particular assay as a monitor of antiviral effect has yet to be established. Thus, while it is tempting to perform all available assays on all trials, it is neither cost-effective nor warranted at the present time. The challenge for the ACTG virologists during the next few years is how to best utilize the increasing number of virologic assays to assess and predict the outcome of a clinical trial. The above compendium is a preliminary attempt to provide some guidelines for the use of these virologic assays as we understand them today. These guidelines will certainly change as new assays are developed and others are validated. All of the assays, with the possible exception of the standard p24 antigen assay, are constantly being revised and improved. Methods are sought to increase ease of use, and to reduce costs, without loss of sensitivity or specificity.

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