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Clinical Use of Quantitative Molecular Methods in Studying Human Immunodeficiency Virus Type 1 Infection

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INTRODUCTION

Several lines of evidence suggest that, at present, the quantitative measurement of human immunodeficiency virus type 1 (HIV-1) load and specific viral gene expression is of major importance in addressing many fundamental questions in the pathobiology of HIV-1 infection and in the clinical management of HIV-1-infected persons (142). For these reasons, the molecular procedures proposed in the last few years for the absolute quantitation of viral nucleic acids in biological samples, their reliability and adaptability to diagnostic applications, and the data obtained by quantitative molecular methods in studying HIV-1 infection are summarized in this article, together with the potential clinical and diagnostic implications of their routine use in virological laboratories.

The peculiar biology of HIV-1 hindered our efforts to understand the links between virus activity and disease onset and development for a long time. Only in recent years has the concept of viral latency (hastily and superficially described by analogy with other viral model systems) been questioned and redefined (69, 126, 127, 141). Moreover, the wide use of PCR-based technology, which boomed in the late '80s and early '90s, contributed to the description of a progressively clearer picture of HIV-1 infection. In fact, the original notion that no HIV-1 activity could be detected in vivo after the acute phase of infection (until the later stages of the disease) was disrupted in some studies in which intracellular transcripts were detected in peripheral blood mononuclear cells (PBMCs) of infected subjects during all stages of disease (9, 32, 148). Soon afterwards, some laboratories demonstrated by reverse transcription-PCR (RT-PCR) the presence of particle-associated, cell-free HIV-1 genomic RNA in plasma samples throughout all stages of infection (8, 15, 120). Furthermore, it was observed that HIV-1 infection is active in lymphoid tissue during the asymptomatic phase of disease (45, 125–129). Although these results suggested a new interpretation of the virological events occurring during clinical latency, more relevant information became available after quantitative analysis of the natural history of HIV-1 infection.

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ROLE OF QUANTITATIVE METHODS

Emerging Features of HIV-1 Infection and Consequent Diagnostic Aspects

Following the isolation of HIV-1 (14) from patients with AIDS or with clinical signs that precede AIDS (2, 7, 30, 47, 108), the typical clinical course of HIV-1 infection was clearly defined; it includes the primary infection, with an acute syndrome of variable severity, a prolonged period of clinical latency, and a final stage of disease characterized by increased susceptibility to opportunistic infections and neoplastic diseases (49, 93). However, great patient-to-patient variability in disease progression was described; in particular, the duration of clinical latency varies widely among infected persons (50, 94), and the progression to AIDS occurs over a median period of 8 to 10 years (22, 92, 95), although immunological and clinical decline is evident much earlier in a significant proportion of patients. Interestingly, the disease does not progress

over an extended time in a small number (approximately 5%) of persons with HIV-1 infection (long-term nonprogressor subjects) (22, 94, 95, 152).

In the last few years, it has been much debated whether $CD4^+$ T-cell depletion and the pathogenesis of AIDS are the result of direct cytolytic effects of HIV-1, T-cell apoptosis by nonspecific activation, dysregulation of cytokine production, or autoimmunity. In agreement with biological, immunological, and molecular evidence, the pathogenesis of HIV-1 infection is depicted, at present, as a multistep process (51); a multitude of viral and host factors potentially involved in infection progression have been studied in the last few years in vitro and in vivo (51, 64, 125, 127, 133, 167). In this complex scenario, several unequivocal biological (3, 100) and molecular (10, 11, 40, 106, 132, 164) findings have emphasized the significant correlation between viral replication and infection progression, including the sharp association between clinical stage and viral load in progressor patients (the vast majority of HIV-1-infected persons) and the particular dynamics of viral activity during the natural course of the disease.

Currently, routine diagnosis of HIV-1 infection is largely based on serological assays, and it is now clear that close to 100% of HIV-1-infected individuals have detectable antibodies to specific viral antigens in their serum, the exceptions being a small proportion of subjects in the earliest stages of the infection. Direct detection of HIV-1 is performed by viral isolation techniques (from PBMCs, plasma, or body fluids), immunological methods for the detection of specific viral antigens (principally HIV-1 p24 antigen in serum or plasma), and molecular methods to reveal the presence of specific HIV-1 RNA and DNA nucleic acids in infected cells, plasma, and body fluids. However, the use of conventional hybridization methods is generally precluded in HIV-1 infection by the low sensitivity of these assays (with the exception of the in situ hybridization methods for some applications), and direct detection of p24 antigen in patient sera is unreliable after seroconversion in most cases because of the small amount of circulating antigen, the presence of bound antibodies, and the low level of sensitivity of this technique.

In the last 10 years, PCR technology has supplied a unique tool for performing both highly sensitive, direct diagnosis of HIV-1 infection (73, 121, 147) and molecular analysis of viral activity in vivo (5, 69, 114) and in vitro (6). Classical and novel PCR applications were used to study HIV-1 infection. One of these new procedures, endogenous RT-PCR (ERT-PCR) (25), is an alternative method to detect cell-free virus in plasma; this technique uses endogenous RT from intact viral particles present in plasma for reverse transcription of genomic HIV-1 RNA to cDNA. Moreover, the presence of small amounts of HIV-1 DNA was demonstrated within HIV-1 virions purified from the plasma of both symptomatic and asymptomatic infected individuals (173) by PCR amplification, suggesting that partial reverse transcription may normally occur within HIV-1 particles. Overall, PCR-based applications provided a specific and efficient alternative for direct detection of HIV-1 infection, in particular for (i) identifying persons who were infected but who had not seroconverted (48), (ii) identifying infected babies born to HIV-1-seropositive mothers, in whom maternal antibodies can persist at detectable levels for 5 to 14 months (137), and (iii) resolving the infection status of subjects with equivocal serological results (74).

More recently, the important evidence that cell-free genomic HIV-1 RNA sequences are detectable in plasma samples of both symptomatic and asymptomatic HIV-1-infected individuals (8, 10, 120, 132) indicated that substantial viral activity may be revealed in almost all patients independent of their clinical condition. These results, obtained by conventional qualitative RT-PCR procedures, emphasized that such assays have several advantages over other methods for analyzing HIV-1 activity and that sensitive quantitative molecular assays were needed for a more precise virological characterization of asymptomatic and symptomatic HIV-1-infected subjects. As a consequence, semiquantitative (53, 54, 106, 119, 151) and quantitative PCR-based methods (for a brief technical review, see reference 34) have been developed in the last few years, allowing the absolute quantitative evaluation of cell-free HIV-1 RNA molecules (viremia) (96, 103, 131, 146), specific viral transcripts in infected cells (10, 103), and proviral DNA sequences in PBMCs (31, 67, 103).

Requirement for Quantitative Methods To Study HIV-1 Infection

Ideally, high sensitivity, specificity, technical flexibility, and adaptability to quantitative analysis are the major features required of a molecular method to evaluate viral activity during most acute or persistent infections (36). This is particularly true for HIV-1 infection, in which detection of very small amounts of viral nucleic acids is necessary for most HIV-1-infected subjects, particularly during the clinical latency phase. In addition, for the precise diagnostic characterization of patients and the reliable monitoring of treated and untreated HIV-1-infected persons, detection and quantitation of different virus-specific nucleic acids, such as genomic HIV-1 RNA, specific viral transcripts in infected cells, and proviral DNA sequences are necessary. Several quantitative studies of viral load during the different clinical phases of HIV-1 infection have been performed recently (10, 11, 27, 106, 129, 132). Although a significant correlation of the average levels of viral load with disease progression was underlined by all investigators, the HIV-1 load appears to be an individual variable in infected persons, as are viral phenotype, organ pathology, and the host's immune response. From a molecular point of view, these quantitative data are in perfect agreement with the results emerging from the analysis of HIV-1 genome variability during the natural course of the infection.

In these latter studies, the potential of the HIV-1 genome to evolve during the infection was analyzed, and it was emphasized that a real mixture of genomically divergent viruses (named quasispecies) (23, 38, 46, 55, 82, 83, 102, 105, 130, 140, 153) is present in infected individuals. This variability of the HIV-1 genome in the form of quasispecies is a major feature of this infection (52); during primary HIV-1 infection, variable regions of the *env* gene are conserved (174, 176), but after seroconversion, sequence diversity expands, yielding viral mutants with distinct biological characteristics (47, 102, 138, 149, 153). The genetic variability enables this virus not only to escape the host's immune response (4, 101, 115) but also to generate drug-resistant viral mutants during therapy (166). It may be hypothesized that, in this infection, the heterogeneous population of viruses is involved in a dynamic race against the host's immune system. Under these conditions, most of the primary biological features of the virus population may change during the infection, including regulatory functions and, consequently, gene expression. Therefore, studies of the balance between viral activity and neutralization by specific immune responses (and their modulation during the different phases of HIV-1 infection) may also help us to understand the pathogenic process and to develop novel strategies for an efficient anti-HIV-1 vaccine.

Viral isolation can only approximate the in vivo situation even when performed quantitatively, and it is reasonable to

believe that in vitro propagation of HIV-1 increases the level of viral quasispecies with high capacity for replication in the cell culture system under use, and thus this method is greatly inaccurate from a quantitative point of view. Furthermore, virus detection by coculture methods is time-consuming, expensive, and more hazardous than nucleic acid detection techniques and thus unsuitable for routine diagnostic applications.

An additional important aspect in medical and diagnostic virology that requires precise, rapid, and easy-to-use quantitative methods is the monitoring of long-term, specific antiviral therapies. Although several obstacles to the wide use of most quantitative molecular methods still remain, the data from recent pilot studies indicate that quantitative techniques for the analysis of viral load in vivo may provide a practical basis for evaluating the effect of antiviral compounds on HIV-1 activity, allowing a more accurate approach to the clinical management of infected patients.

QUANTITATION OF SPECIFIC VIRAL DNA AND RNA SEQUENCES

Attempts To Quantify Nucleic Acid Species in Biological Samples

In the last few years, PCR-based and non-PCR methods were planned, developed, and used to quantify specific DNA and RNA species present in small amounts in biological samples. These novel procedures were proposed as alternatives to conventional molecular hybridization methods (i.e., Southern and Northern [RNA] blots, dot hybridization, and spot hybridization), since these methods, while allowing semiquantitative evaluation of nucleic acid species, show a low level of sensitivity in most virological applications.

Recently, a branched hybridization procedure, branched DNA (bDNA), with increased sensitivity as a result of signal amplification has been developed (160) and applied to directly quantifying cell-free HIV-1 genomic RNA molecules in serum samples (39). In this procedure, viral particles present in plasma are incubated with lysing buffer and specific HIV-1 probes, and the viral RNA-probe complex is then incubated in microplate wells in the presence of a second (solid-phase-adsorbed) capture probe. Subsequently, the immobilized target is hybridized by using multiple copies of probes labeled with alkaline phosphatase and then incubated with dioxetane, a chemiluminescent substrate. Finally, light emission is measured by a luminometer and compared with the data obtained with standard positive samples with different concentrations of HIV-1 RNA sequences. The bDNA assay system was used to study HIV-1-infected patients under therapy with specific antiviral compounds (27, 39, 161). In these applications, it was observed that this method is simple to perform and potentially able to be used in routine applications. However, several aspects presently limit its use in routine studies of HIV-1 infection, and the accuracy of the quantitation obtained still requires thorough evaluation. Since amplification is accomplished by the incorporation of sites for 1,755 alkaline phosphatase-labeled probes per genome of HIV-1 in bDNA (122), in theory, small differences in hybridization efficiency may lead to great differences in the quantitative data, depending on the signal amplification procedure used in the assay. Moreover, various issues of performance and other features of bDNA-based assays should be significantly improved before this method is introduced widely into diagnostic virology. Among these features are the assay sensitivity (currently, the bDNA detection threshold for HIV-1 RNA sequences in plasma or serum is very high, 5×10^2 molecules per ml in the last generation of the assay) (27, 72) and the flexibility of the method (an ideal diagnostic technique should be able to quantify different specific HIV-1 nucleic acids in biological samples, i.e., different classes of viral transcripts, cell-free genomes, and proviral molecules) (72).

In the past few years, RNA and DNA amplification procedures other than PCR have been developed (66, 84, 169). Attempts to quantify nucleic acids in biological specimens have been carried out by these amplification methods; one of these techniques, nucleic acid sequence-based amplification (NASBA), is carried out isothermally with a mixture of three enzymes: avian myeloblastosis virus reverse transcriptase, T7 RNA polymerase, and RNase H. This assay was used specifically to detect HIV-1 RNA molecules in plasma samples and HIV-1 transcriptional activity (21) in PBMCs. Recently, a quantitative adaptation of this technique that uses internal standards and an electrochemiluminescent detection system was developed to determine the level of HIV-1 RNA molecules in plasma samples from HIV-1-infected individuals (162).

Several quantitative and semiquantitative PCR-based strategies were also proposed. In particular, methods were developed for quantitation of the amplification product (81, 86, 119, 163, 165); a rationale for these applications was supplied by the evidence that there is a linear relationship between the input template and the amount of amplification product in PCR assays (54, 154). However, since the linear relationship is maintained only for initial amounts of DNA or RNA within a limited range, these procedures may be greatly imprecise when samples containing highly variable amounts of nucleic acids are examined, as is frequently the case when clinical specimens are analyzed. Furthermore, any difference in amplification efficiency may greatly influence the quantitative evaluation of products, particularly when the initial amount of the template is small or the template is incompletely purified. These differences may depend on sample preparation, purification of nucleic acids, the presence of inhibitors, and machine performance and are unpredictable in most cases. For similar reasons, reliable quantitation of DNA and RNA species by PCR and external standard reference curves (17) or limiting dilution of samples is not warranted.

Competitive PCR-Based Methods

An increasing number of technical studies and pilot applications indicate that, at present, competitive PCR (cPCR) and competitive RT-PCR (cRT-PCR) methods are the procedures of choice for quantitative detection of nucleic acid species in biological samples (10, 35, 36, 42, 62, 103, 107, 131, 132, 146) and that these methods allow precise and reliable evaluation of the mean viral activity rate in real time during the different clinical phases of HIV-1 disease.

The competitive method is based on coamplification of two similar template species (16), the wild-type sequence to be quantified and the reference template introduced in a known amount; these templates have equal or similar lengths and share primer recognition sites. Under these conditions, the similar templates compete for the same primer pair during amplification (cPCR), thus amplifying at the same rate independent of the number of cycles and of any variable influencing the PCR amplification (42) (i.e., any predictable or unpredictable variable has the same effect on both template species). The amount of final product is calculated differently in different applications of the technique (16, 42, 70, 103), but regardless of the method used, the ratio of these products precisely reflects the ratio of the initial concentrations of both template molecules (35).

The choice of an appropriate DNA or RNA competitor template has a key role in competitive methods and requires the utmost care. In fact, the characteristics of these modified sequences ensure identical thermodynamics and amplification efficiency for both template species used in competitive reactions. The function of PCR amplification, $Y = Y' (1 + e)^n$, where Y is the product yield, Y' is the starting sequence copy number, e is the amplification efficiency, and n is the number of cycles, in cPCR is as follows:

$$C/W = C' (1 + e)^n/W' (1 + e)^n = C'/W'$$

where C and W are the product yields of the competitor and wild-type templates, respectively, and C' and W' are the initial amounts of both template species, respectively (34, 42). Since e and n are identical for both templates in cPCR (according to the general concept of this reaction), the relative product ratio C/W depends directly on the ratio of the initial concentrations of the two DNA species (C'/W').

Generally, different concentrations of competitors are challenged in different tubes against a constant amount of wild-type template in order to cover the possible range of wild-type concentrations. This is necessary in all virological applications in which clinical specimens with highly divergent concentrations of the wild-type sequence are assayed. Recently, the successful application of the competitive method to HIV-1 DNA and RNA species was described; after extensive optimization, the wild-type template and a fixed amount of competitors were amplified in a single tube (500 copies of HIV-1 DNA or 1,000 copies of HIV-1 RNA) (67). This interesting study indicates the possible development of fast, cheap, and simple competitive methods for wide diagnostic use in the near future.

Competitors identical in size to the wild-type template but bearing a novel restriction site were used in cPCR applications (78, 110). In this procedure, amplification products are recognized by digestion with the specific restriction enzyme followed by gel electrophoresis. This strategy, however, should be avoided, since an additional step (enzyme digestion) whose efficiency may vary unpredictably is introduced and quantitative analysis of digested amplification products by conventional procedures may be imprecise. Competitors that are identical in size but different in internal sequence may be needed in some applications, however, such as when capture probes are used to separate the cPCR products (18). In these cases, careful attention is necessary to verify the influence of each internal modification on the amplification efficiency as well as the hybridization efficiency of capture probes.

Competitors with different lengths than the wild-type sequences have been used extensively in cPCR. In these cases, competitor DNA templates may be generated rapidly by PCR-mediated insertion or deletion mutagenesis (56, 75), quantified, and used directly or after cloning in appropriate vectors. RNA competitors may be obtained by transcription in vitro of mutated DNA fragments after cloning in appropriate expression vectors (65, 103, 131). Of note, an advantage of using competitors that differ in size from the wild-type template is that the amplified products can be recognized and quantified directly after simple gel electrophoresis and ethidium bromide staining. However, it was emphasized (35) that great diversity in sequence length (greater than 15%, in our experience) may generate significant differences in amplification efficiencies; for this reason, internal insertions or deletions should be reduced to the minimum necessary for obtaining discrete bands of the PCR products by gel electrophoresis.

Recently, a replication-competent HIV-1 mutant with a 25-bp insert was generated and used as an internal control in an application of the competitive strategy (116). With this mutant virus, which is added to the sample in a known amount before RNA extraction, the possible variability of RNA recovery during purification should be normalized, thus increasing the accuracy of the competitive assay. This study indicates that development of reproducible procedures to evaluate the loss of viral nucleic acids during purification is possible. It should be underlined, however, that precise quantitation of competitor DNA and RNA concentrations is always required in competitive methods. Quantitative analysis of competitor concentration is generally achieved by gel electrophoresis, endpoint dilution amplification by spectrophotometry, and Poisson analysis of the last dilution that gives positive values after PCR or RT-PCR under optimal conditions. The quantified competitor should also be challenged against a fixed amount of the wild-type template, previously quantified by endpoint dilution and Poisson distribution, by cPCR or cRT-PCR for a further cross-check.

For competition analysis, densitometric evaluation of ethidium bromide-stained gels makes the final step easy, fast, and cheap; gels are generally scanned with a densitometer either directly (by positive fluorescent emission on the transilluminator) or after photography. The peak areas of both amplified products may be calculated, but when a deleted competitor or a competitor with a small insertion is used, the corresponding value has to be corrected for the different levels

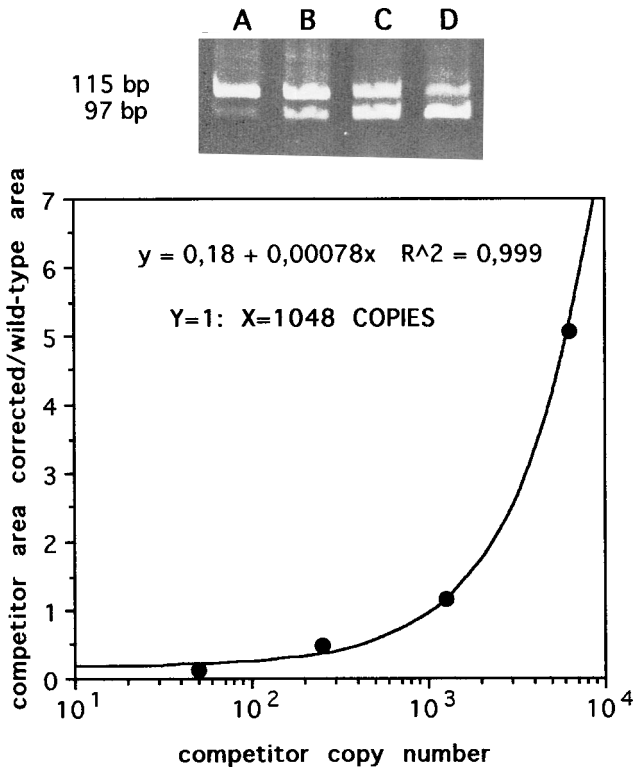

FIG. 1. Quantitative analysis of HIV-1 RNA copy numbers in plasma samples by cRT-PCR. Gel electrophoresis of coamplification products is shown above the graph. Lanes (left to right) contain increasing copy numbers (50 to 6,250) of competitor RNA (97 bp) and a fixed amount of wild-type RNA (equivalent to the amount in 100 μl of plasma) (115 bp). The graph represents densitometric analysis of the ratio between the peak area of the deleted band (corrected for the lower ethidium bromide incorporation) and the wild-type band (y axis) plotted against RNA competitor copy number (x axis). The number of wild-type RNA molecules is deduced by calculating the equivalence point for the ratio of the competitor area to the wild-type area ($y = 1, x = 1,048$).

of ethidium bromide incorporation (Fig. 1) (103). It has been suggested that capillary electrophoresis may be a suitable method for a correct analysis of cPCR products (156). More recently, simplified procedures with capture probes (18, 163, 172) were proposed with the aim of simplifying detection of amplicons and analysis of competitive reactions. While these methods are accepted in most diagnostic virology laboratories, since the final phase of the reaction is shortened, they introduce unnecessary additional steps to cPCR, thus reducing, in our opinion, the precision and reliability of molecular quantitation.

THE NATURAL HISTORY OF HIV-1 INFECTION

Primary Infection with HIV-1

Since the first events following initial contact with HIV-1 cannot be followed very closely in humans, a significant proportion of the studies aimed at defining the relevant aspects of this early phase of the infection with HIV-1 or different lentiviruses in animal models.

After mucous membrane inoculation of simian immunodeficiency virus (SIV) in monkeys (109), infection is first localized in draining lymph nodes before systemic spread (28, 143). This process may last a few weeks in humans before HIV-1 colonizes lymphoid tissue in the entire body and appears in the circulation before seroconversion.

By this time, the virus can be isolated readily in cell-free plasma and is usually of the non-syncytium-inducing (non-SI) phenotype (138). The numbers of peripheral $CD4^+$ T cells drop in all subjects at this early phase of the infection but to very different extents. The molecular parameters of viral replication vary in different infected individuals during the primary infection but generally peak at high values: plasma viremia (cell-free genome copy number, as measured by cPCR) is approximately 10^6 copies per ml of plasma (11, 33, 113). In our experience, one subject had 2×10^7 HIV-1 RNA copies per ml of plasma, while a pregnant woman who seroconverted after 5 months of pregnancy revealed a biphasic peak of viremia. The second peak appeared after delivery but never reached 3,000 copies per ml of plasma in the samples available to us (13). During the primary infection with HIV-1, the dynamics of specific viral intracellular transcripts and proviral DNA virtually parallel plasma viremia (11). Notably, the molecular indexes of viral replication are highest in patients whose primary infection appears as a transient AIDS-like syndrome, with a dramatic drop in $CD4^+$ T-cell counts.

After the initial burst of viral activity (63), within a few weeks a strong immune response reduces all viral indexes to what will be the lowest levels ever for each infected subject except during specific anti-HIV-1 therapy. In parallel, $CD4^+$ T-cell counts recovery partially and sometimes totally, while $CD8^+$ T-cell levels usually remain elevated. Although a substantial drop in cell-free virus is documented in all cases, complete clearance of HIV-1 from plasma does not occur in the natural course of the acute infection (33).

Recently, a parallel analysis of viral load and HIV-1 phenotype was performed with serial samples from infected patients (seroconverters) who progressed to AIDS within 5.5 years (77). The data were compared with those for samples from persons who remained asymptomatic for more than 5.5 years after HIV-1 infection. At seroconversion, virus load was not significantly different in the two groups, but a significant decline in HIV-1 RNA copy numbers was observed during the early period of the infection in serum samples from the slow progressors compared with the progressor patients. Although these and other results from the study of HIV-1 phenotype and viral load and their correlation with infection progression (149) strongly suggest that the degree of viral replication during the early phase of HIV-1 infection may have a prognostic meaning, further analysis is necessary to evaluate the role of HIV-1 phenotype in disease progression. While it was observed that the appearance of SI variants may be a useful prognostic marker for decline in $CD4^+$ T-cell counts and rapid progression to AIDS, SI variants are not required for disease progression and are undetectable in about 50% of AIDS patients (136, 142). In this context, studies of the early stages of SIV infection in macaques (29, 134, 150) may be useful to address questions of the relationships between retroviral load and host immune response and their influence on infection progression.

Clinical Latency

Many independent researchers have investigated the asymptomatic phase of HIV-1 infection and studied infected subjects by different laboratory methods. The data accumulated trace a pattern of the evolution of the disease. A few cross-sectional studies (11, 106, 132) have pointed out that individual variability of molecular indices is the rule in this period but a good inverse correlation can be established between these indices and $CD4^+$ cell counts. As documented in the past few years (45, 124, 126), most viral activity during clinical latency is localized in the lymphoid organs throughout the body, where target cell concentration, recruitment, and activation by accessory cells (together with immune complex formation and virion trapping) constitute the ideal milieu for active HIV-1 replication. These studies confirmed and extended previous investigations (57, 123) indicating that lymphoid germinal centers are efficient reservoirs of HIV-1 and documented that viral expression is generally 100 to 500 times greater in lymph node cells than in PBMCs, accounting for more than 99% of total virus load in the body. Although a strong correlation exists between levels of specific viral transcripts in lymph node cells and PBMCs (129), the transcriptional activity of each provirus must be much higher in lymph node cells than in PBMCs, since the difference in proviral concentration is not as striking (two to three times). Furthermore, recent evidence (166) indicates that HIV-1 viremia is sustained by a dynamic process involving continuous rounds of virus replication and de novo infection; in other words, most cell-free virus in the plasma of HIV-1-infected patients derives from short-lived, actively replicating cells rather than chronically infected (activated or not activated) PBMCs.

Soon after primary infection, all indices of viral activity are generally low. Proviral DNA sequences can be detected with very sensitive PCR procedures in 10^5 PBMCs (average, 10^2 HIV-1 DNA copies per 10^5 PBMCs). Plasma viremia can be as low as 200 viral genomes per ml of plasma, the average being 10^4. At this stage, viral isolation from plasma is difficult because of good neutralizing activity in serum, although it is generally possible to recover the virus by cocultivation of lymph node mononuclear cells (LNMCs) with phytohemagglutinin (PHA)-stimulated blasts, suggesting that neutralizing antibodies do act to limit HIV-1 replication during the lengthy asymptomatic stage of infection (24).

The clinical latency phase can last for several years (median, 10 years) (92), but its length is extremely variable among individuals. During these years after primary infection, the equilibrium established between viral replication and immune response is gradually shifted towards an increase in the former and a weakening of the latter. Immune deterioration, monitored by the decrease in $CD4^+$ T-cell counts, is accompanied

by loss of helper function and by histopathological changes in the lymphoid organs (124, 127, 128).

A few studies (11, 53, 106) have shown that this evolution tends to be rather slow at the beginning and accelerates later on, following a typical curve. In Fig. 2, we have summarized and merged the results from 12 HIV-1-infected progressor patients to illustrate the pattern of the dynamics of viral molecular indices during infection progression.

Disease Progression

As may be noted, the patterns of the parameters of HIV-1 activity shown in Fig. 2 are exponential, and immunodeficiency (in terms of the drop in $CD4^+$ T cells) rises in parallel. The acceleration of infection progression has always been a matter of debate; attempts to establish a link between this phenomenon and so-called cofactors that might determine its slope in vivo have so far been inconclusive despite the great deal of research performed in vitro providing evidence of modulation of HIV-1 transcriptional activity in the presence of eukaryotic or viral heterologous factors (64).

Some researchers tend to identify a threshold in the molecular indices of viral activity as a general marker for malignant and irreversible progression. However, two crucial aspects call for more detailed analysis of the relationship between viral load and disease progression. First, patient-to-patient variability in the parameters of HIV-1 activity is such that totally asymptomatic carriers of HIV-1 often appear to be more heavily infected than are some AIDS patients, and in this situation, only individual follow-up might reveal upregulation of viral activity. Second, the increase in viral activity in most cases is not so sharp as to suggest a sudden pathogenetic event but rather seems to be gradual. Nevertheless, recent studies (71, 166) have nicely demonstrated that HIV-1 infection is far from being a typical lentiviral infection; the turnover of infected cells and of circulating virions is quite rapid during the infection. These findings have highlighted the fundamental fact that, independent of the biopathology of $CD4^+$ cell killing (viral or immune cytotoxicity), cell death is strongly linked to viral replication. The death of $CD4^+$ T cells poses a serious challenge to their replacement; the evolution of the infection might well be viewed as a race between cell destruction and replacement.

Long-Term Nonprogressor Patients

A small fraction (probably fewer than 5%) of the HIV-1-infected population not only displays a longer than average asymptomatic infection but also seems to maintain a high and stable number of $CD4^+$ T cells during this time. These subjects have been designated long-term nonprogressors (LTNPs). Although the distinction between the LTNP population and the very slowly progressing subjects seems difficult at present, a great deal of interest has been aroused by this subgroup of HIV-1-infected persons in the hope of identifying the factor(s) that determines their ability to escape the effects of HIV-1 infection.

In two recent works (27, 129), the virological and immunological parameters of infection of these LTNP subjects have been thoroughly compared with those of progressors. Although molecular data were obtained by different techniques, both studies showed a clear and significant difference in virological parameters between the two groups of patients, as shown in Table 1. However, Cao and coworkers (27) did not observe any difference in growth kinetics between isolates from nonprogressors and asymptomatic progressors. The immune response was also investigated, and both studies (together with data from an earlier work [95]) agree in finding more efficient humoral and cellular responses in LTNPs than in progressors. However, since these studies used control populations with low $CD4^+$ T-cell numbers, these findings are not definitive as yet.

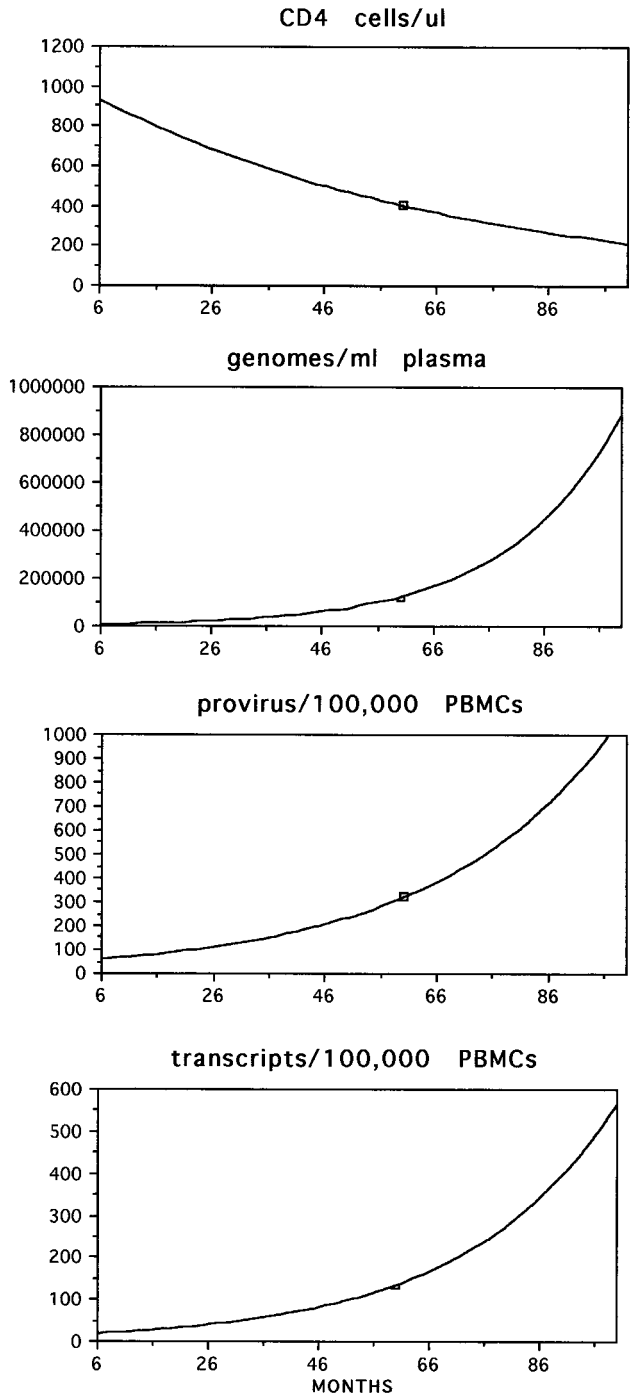

FIG. 2. Pattern of the dynamics of molecular parameters during clinical latency and disease progression. Quantitative sequential data from 12 HIV-1-infected progressor patients monitored from clinical latency to AIDS were combined, and mean dynamic curves for HIV-1 genome copy number in plasma, number of HIV-1 proviruses per 100,000 PBMCs, and number of HIV-1 transcripts per 100,000 PBMCs were calculated in parallel with $CD4^+$ T-cell counts. The *y* axis shows (from top panel to bottom panel) $CD4^+$ T cells per microliter of blood, HIV-1 genome molecules per milliliter of plasma, provirus copy number per 10^5 PBMCs, and transcript (unspliced) molecules per 10^5 PBMCs. The *x* axis shows months after primary HIV-1 infection.

TABLE 1. Cross-sectional analysis of the molecular parameters of HIV-1 activity and replication in LTNPS and progressors[a]

Patients	No. of HIV-1 genomes/ml of plasma	Avg no. of copies/10^6 PBMC		Avg no. of copies/10^6 LNMCs	
		HIV-1 proviral DNA	Unspliced mRNA	HIV-1 proviral DNA	Unspliced mRNA
LTNPs	70,818	638	1,072	2,194	743,270
Progressors	1,586,967	4,937	10,450	11,394	2,590,080

[a] LTNPs had a stable number of $CD4^+$ T cells (>600/μl of blood) for more than 7 years with no antiviral therapy. Progressor patients had $CD4^+$ T-cell counts ranging between 400 and 200/μl of blood. For more detailed analysis of the data, see reference 129.

Interestingly, additional data obtained by one of us (104) point out that as far as levels of quantitative plasma RNA, PBMC transcripts, and proviral DNA are concerned, no clear difference can be identified when the nonprogressor group of individuals is compared with a group of subjects with high $CD4^+$ T-cell numbers. As most of the latter group are bona fide progressors, the emerging consideration is that nonprogressor subjects might just remain "frozen" in very early stages of the infection and that some as yet to be identified factor(s) allows them to evade further evolution of disease. Ongoing studies are investigating the immune response and the variability of the virus in order to identify some peculiarity in LTNPs that might render them refractory (at least for a prolonged time) to HIV-1 pathogenicity.

MONITORING THERAPY WITH SPECIFIC ANTI-HIV-1 COMPOUNDS

Ideal Parameter To Monitor Efficiency

Research progress in the understanding of HIV-1 biology and virus effects on the human immune system is yielding new ideas for conventional and novel strategies for halting HIV-1 replication and its consequent damage to the immune system of infected persons. Presently, many clinical trials to test different anti-HIV-1 treatments are under way (1). The addition of new agents to the antiretroviral armamentarium will expand the treatment options available to clinicians who treat patients with HIV-1 infection. In this context, methods aimed at evaluating the efficacy of therapies in real time (allowing close monitoring of long-term drug therapies with antiretroviral agents) permit treatment to be tailored for specific needs (43).

To monitor anti-HIV-1 therapies efficiently, a major aspect is the choice of the best parameter to be used for evaluating viral activity frequently and precisely. In the last 10 years, several indirect parameters (named surrogate markers) have been proposed to study disease progression and to monitor antiviral therapies (85, 158, 159). Although some aspects of this study may be of interest from a clinical and pathogenetic point of view, the use of these indices does not allow reliable analysis of viral replication in HIV-1-infected patients under treatment.

Among the virological parameters of viral replication, the quantitative detection of HIV-1 p24 antigen in plasma was used as a direct index of virus replication and applied to clinical monitoring of anti-HIV-1 therapies. However, this immunological method has a low sensitivity level even if circulating p24–anti-p24 immune complexes are dissociated by using a buffer with low pH (111, 145). More recently, the significance of the HIV-1 transcriptional profile during disease progression was addressed (107, 144). Of interest, it was suggested that disease progression is associated with (or, in some cases, even predicted by) a rise in all HIV-1 transcripts, in particular, in multiply spliced mRNAs encoding *tat*, *rev*, and *nef* products. In other cases, clinical evolution was accompanied by a shift in the balance of HIV-1 mRNAs from multiply spliced to unspliced messengers. However, the biological meaning of the variability in the HIV-1 transcription pattern during infection is still controversial, and further evaluation is required to define whether the relative ratio between unspliced and spliced mRNAs supplies reliable prognostic information and consequently may be employed as a parameter useful for monitoring HIV-1-infected patients under antiretroviral treatment.

On the other hand, recent studies (71, 166) provided clear evidence that virus turnover occurs sooner in plasma than in PBMCs. This information may be a key for the correct monitoring of specific anti-HIV-1 therapies. In a recent follow-up study of HIV-1-infected individuals treated with nucleoside analogs (12), we evaluated the dynamics of different quantitative parameters of viral activity (number of cell-free HIV-1 RNA molecules per milliliter of plasma, specific viral transcript copy numbers in PBMCs, and provirus copy numbers in PBMCs). The effectiveness of antiviral drugs on viral replication was shown by significant reductions in viremia levels and by a parallel decrease in the RNA/DNA ratio (mean transcriptional activity; HIV-1-specific transcript copy number/provirus copy number per 10^5 $CD4^+$ PBMCs). In a few studies, viral load was analyzed in parallel with the HIV-1 genotype of plasma virions (12, 71, 166), confirming that cell-free viruses showing mutations that confer drug resistance are easily detectable in plasma samples when a rebound of viral load is observed in treated patients. These data, together with the observation that the amount of cell-free virus in plasma (determined by molecular methods) reflects better than any other parameter (namely, provirus copy number or specific viral transcript molecules in PBMCs) what happens in different cell or tissue compartments, suggest that, at present, quantitative assay of plasma viremia is the most reliable method for studying HIV-1-infected patients under treatment.

Antiretroviral Therapies and Analysis of HIV-1 Load

A group of powerful anti-HIV-1 compounds include inhibitors of virally encoded enzymes (79) necessary for successful completion of the viral life cycle. After the first nucleoside analog (zidovudine; 3′-azido-3′-deoxythymidine [AZT]) was introduced into clinical use, other anti-HIV-1 nucleoside compounds were rapidly made available to treat infected patients, including didanosine (ddI), dideoxycytosine (ddC), and stavudine (d4T); these compounds all target the virus-specific enzyme RT. More recently, inhibitors of the HIV-1 protease (compounds designed to interfere with the specific protein-processing enzyme of HIV-1) have been developed and proposed for anti-HIV-1 therapy, and many new drugs are in limited experimental use or approaching clinical application.

During therapy, mutation of HIV-1 to drug resistance is a major obstacle to virus containment (19, 20, 87, 157), and this may account for the transitory immunological improvement in treated patients. The use of AZT in patients was monitored, and it was observed that HIV-1 strains isolated from subjects during long-term treatment with AZT showed reduced suscep-

tibility to the drug (19, 87); furthermore, it was observed that the changes in viral phenotype of AZT-resistant viruses were associated with several mutations at five codons in the *pol* gene of HIV-1 (80, 88). The association noted between the degree of AZT resistance and the number of specific mutations in the *pol* gene of HIV-1 has provided the basis for using molecular assays to assess drug susceptibility (89, 117), and similarly, it was observed that particular HIV-1 variants become resistant to non-nucleoside inhibitors of RT (26, 118, 135) and protease inhibitors (99) by specific mutations. Moreover, gene therapy represents a promising alternative to conventional treatments for HIV-1 infection. In these approaches, antisense oligonucleotides, ribozymes, RNA decoys, transdominant mutants, toxins, and immunogens are used (44), supplying a powerful means to target multiple sites of the HIV-1 genome (58, 97, 139, 170, 171, 175). In particular, RNA decoys are short RNA oligonucleotides that mimic the HIV-1 transactivation response element (TAR) or Rev response element (RRE), which act by inhibiting the binding of the HIV-1 regulatory proteins Tat and Rev to the true TAR and RRE regions of the HIV-1 RNA. Recently, cultured cells were durably protected from HIV-1 infection by using an RRE decoy (91), suggesting that RNA decoys are promising candidates for use in vivo as antiviral molecules for gene therapy against AIDS. However, gene therapy strategies require not only precise identification of the genes that effectively inhibit HIV-1 replication but also efficient vector systems for viral and nonviral gene delivery (168) and expression in vivo. Reliable laboratory methods to evaluate viral activity are needed as well.

Two recent studies (71, 166) used a quantitative approach and focused on the dynamics of HIV-1 infection. They confirmed that this virus has a great potential to evolve in response to the selective pressures exerted by specific antiviral compounds. Overall, these findings have suggested that, in HIV-1 infection, viral activity, cell death, and cell replacement are in balance during the clinically latent phase of the infection (37). In particular, when cycles of HIV-1 infection are interrupted by potent antiviral therapy, HIV-1 levels in plasma drop dramatically in most patients (12, 166), and this drop is associated (at least in some cases) with reciprocal increases in $CD4^+$ lymphocyte counts. These immunological responses, however, are of limited duration, returning to baseline or even to higher values within a few weeks or months. Of note, sequence analysis of viral RNA present in plasma demonstrated that, along with the rebound of viral load in treated patients, mixtures of wild-type viruses and drug-resistant HIV-1 mutants are frequently detectable (12, 166), demonstrating that a process of selection of functionally active (drug-resistant) viruses is active in this phase. Importantly, these studies indicated unambiguously that the dynamics of HIV-1 load during treatment parallel the process of selection of drug-resistant viral mutants in most cases. These studies also suggested that quantitative methods are sensitive enough to monitor antiviral therapies and to provide information on changes in viral activity (Fig. 3). It was also observed that the decline in cell-free virus in blood has a half-life of about 48 h (71, 166); of note, these results suggest that about 30% of the virus population in plasma is replaced daily and illustrate the importance of quantitative methods to understand the pathobiology of HIV-1 infection. Several outstanding features of these results call for more detailed analysis to explain, in particular, the rise in $CD4^+$ T-cell counts following treatment with potent anti-HIV-1 agents ($CD4^+$ T-lymphocyte redistribution or cell proliferation?) (41, 112, 155). However, these findings may have a central role for our understanding of both AIDS pathogenesis and the dynamics of the effects of antiviral agents in vivo.

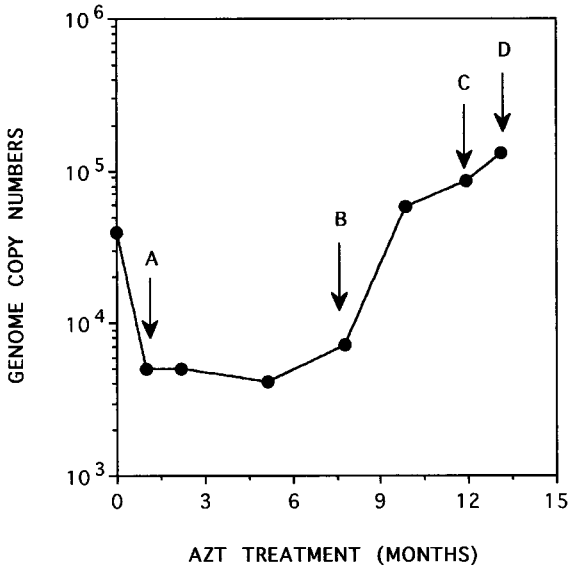

FIG. 3. HIV-1 genome copy numbers in plasma during AZT treatment and direct sequencing of the HIV-1 gene *pol* from cell-free plasma virions. HIV-1 viremia was determined by cRT-PCR. A 769-bp fragment of the HIV-1 *pol* gene (in which mutations relevant to the development of AZT resistance occur) was amplified (at the time points indicated by arrows) with specific primers and sequenced (12) to study the RT genotype at codons 41, 67, 70, 74, 215, and 219. In samples from this patient, a wild-type *pol* genotype was detected at time points A and B, while mutations at positions 67 (Asp→His) and 70 (Lys→Arg) relevant to the development of resistance to AZT were detected at time points C and D.

Moreover, the results obtained in these studies strongly support the hypothesis that $CD4^+$ T-lymphocyte depletion is principally dependent on cell killing by virus- and immune system-mediated effects rather than a lack of cell production (71).

However, the rapid generation and selection of drug-resistant HIV-1 mutants is considered a major clinical challenge. The viral turnover drives both the pathogenic process and the development of genetic variation of HIV-1; under these conditions, accumulation of viral variants resistant to the antiviral compounds under wide clinical use and the consequent presence of these variants even before therapy are predictable (37). A possible strategy to overcome this problem is to administer combinations of antiviral agents that target different virus-specific proteins or functions; the rationale for combining anti-HIV-1 agents is to provide more efficient viral suppression by means of the synergistic effects of several promising anti-HIV-1 compounds (61, 68, 98) and to limit the emergence of drug resistance during prolonged drug administration (76). However, careful attention should be devoted to the possible increased toxicity of combination therapy (59). Clearly, the opportunity of treating HIV-1-infected individuals by complex pharmacological strategies calls for unequivocal means to monitor the efficacy of the compounds in vivo, emphasizing the potential impact of quantitative molecular methods in clinical virology.

Overall, the aspects discussed above, together with the results from some applications of quantitative molecular methods to the monitoring of anti-HIV-1 therapies, might have practical implications for the medical management of infected subjects. Powerful molecular tools have opened a new way of studying HIV-1-infected patients under therapy with specific antiviral compounds; furthermore, the availability of quantitative assays to measure viral load in real time may allow not only more rational use of most of the clinically licensed drugs than

before but also more efficient and rapid evaluation of the efficacy of new compounds or novel strategies for treatment.

CONCLUDING REMARKS

While a few authors still suggest the usefulness of HIV-1 proviral load as an efficient molecular index of HIV-1 activity and infection progression (17, 164), the majority of complete quantitative studies have repeatedly indicated that the analysis of cell-free genomic RNA copy number in plasma has a key role in evaluating this activity in vivo, since the amount of cell-free virus in blood directly reflects the level of HIV-1 expression in different cells and tissues during infection.

It was recently observed (141) that understanding of the immunopathogenesis of HIV-1 infection increased significantly when HIV-1 activity in lymph nodes was appreciated and advanced virological and molecular methods for accurate analysis of viral load were introduced. Although $CD4^+$ T-cell counts still remain an important index for evaluating disease progression in HIV-1-infected patients, more direct and quantitative measures of viral activity supplied a clearer picture of the natural history of this infection. Furthermore, most of the natural history studies of HIV-1 infection substantiated the clinical rationale for early diagnosis and treatment, providing a theoretical and practical basis for the assessment of therapeutic strategies by dynamic measurement of viral load (142).

In the previous sections, we have underlined the theoretical and practical aspects indicating that cPCR-based procedures are currently the techniques of choice for quantifying nucleic acid species present in small amounts in biological samples. Quantitative cPCR and cRT-PCR procedures, carried out by challenging a known amount of competitor RNA or DNA sequence against a wild-type nucleic acid species to be quantified, are independent of most of the predictable and unpredictable variables affecting qualitative PCR procedures (42).

However, to use cPCR and cRT-PCR methods in routine clinical applications to their fullest potential, several technical steps should be simplified or optimized in the near future (60). First, international standard samples for quantitative molecular procedures are currently necessary to evaluate the data obtained by different methods; second, efficient and reproducible procedures for sample preparation and RNA and DNA purification and methods for easy identification and reliable measurement of amplification products should be developed to increase the precision of quantitative methods; third, development of easy-to-use techniques for detecting and quantifying amplification products (90) may be important to minimize cumbersome and time-consuming procedures, reduce costs, and increase productivity. However, it should be evident that an efficient and sensitive detection assay system cannot compensate, in PCR applications, for poor amplification.

Overall, different virological, molecular, and technical aspects summarized here suggest that the availability of quantitative methods may significantly increase the diagnostic potential of medical virology laboratories in studying patients with HIV-1 infection. Natural history and pathogenicity studies carried out directly in vivo have depicted a general profile of HIV-1 activity during the different clinical phases of the infection. A common conclusion of these studies was that early treatment of HIV-1-infected persons might be important in retarding, or even blocking, disease progression. These factors highlight the need for laboratory methods to monitor HIV-1 patients under therapy and indicate that second-level laboratory facilities will be required in the near future to answer the questions generated by conventional and novel treatments. Although further technical improvement and optimization are clearly necessary for large-scale use of the quantitative molecular methods described here, reliable quantitation of cell-free HIV-1 RNA molecules and specific virus transcripts for clinical applications is far from being a mirage; in fact, there is a real opportunity for further development.

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