EXHIBIT I

CLINICS IN LABORATORY MEDICINE

HIV/AIDS

ROGER J. POMERANTZ, MD, GUEST EDITOR

VOLUME 14 • NUMBER 2 • JUNE 1994

W. B. SAUNDERS COMPANY
A Division of Harcourt Brace & Company
PHILADELPHIA LONDON TORONTO MONTREAL SYDNEY TOKYO

W. B. SAUNDERS COMPANY
A Division of
Harcourt Brace & Company

The Curtis Center
Independence Square West
Philadelphia, PA 19106-3399

The *Clinics in Laboratory Medicine* is covered in *Excerpta Medica, Index Medicus, Current Contents/Clinical Medicine, BIOSIS,* and *ISI/BIOMED.*

CLINICS IN LABORATORY MEDICINE **ISSN 0272-2712**
June 1994 Volume 14, Number 2

The *Clinics in Laboratory Medicine* (ISSN 0272-2712) are published quarterly by W. B. Saunders Company. Corporate and Editorial Offices: The Curtis Center, Independence Square West, Philadelphia, PA 19106-3399. Accounting and Circulation Offices: 6277 Sea Harbor Drive, Orlando, FL 32887-4800. Second class postage paid at Orlando, FL 32862 and additional mailing offices. Subscription price is $77.00 per year (U.S. individuals), $94.00 per year (U.S. institutions), $103.00 per year (foreign individuals), and $109.00 per year (foreign institutions). Foreign air speed delivery for all *Clinics* is $6.00 per issue. All prices are subject to change without notice. POSTMASTER: Send address changes to the *Clinics in Laboratory Medicine,* W. B. Saunders Company, Periodicals Fulfillment, Orlando, FL 32887-4800.

Customer Service: 1-800-654-2452.

The editor of this publication is Sandra W. Hitchens, W. B. Saunders Company, The Curtis Center, Independence Square West, Philadelphia, PA 19106-3399.

Printed in the United States of America.

CON

THOMAS
Cincin

GUES

ROGER J.
Retro
Philad

CONT

OMAR BA
Depar
Philad

ROBERT
Labora
Washi

RICHARD
School
Hospit

HOMAYO
Public

FRANÇOI
Respor

BENJAMI
and Ex
Massac

MARTIN S
Unit, M
Massac

MARK HO
Departi
Profess

CONTRIBUT

HIV/AIDS 0272-2712/94 $0.00 + .20

CLINICAL APPLICATION OF REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION FOR HIV INFECTION

Mark Holodniy, MD

Gene amplification was first described in 1985 using a thermolabile *Escherichia coli* Klenow fragment.[46] Three years later in 1988, a thermostable DNA polymerase was described that could withstand multiple cycling temperatures and, thus, no longer required the addition of further DNA polymerase to allow a reaction to be completed.[55] In 1987 the polymerase chain reaction (PCR) was described for cellular DNA containing HIV proviral DNA.[36] In 1988 a report described the ability to detect intracellular RNA from HIV-infected cell lines using reverse transcription (RT) followed by PCR.[10] This was quickly applied to HIV-infected patient material in which RT-PCR was used for the detection of mRNA in peripheral blood mononuclear cells (PBMCs)[20,47] and virion-associated RNA in serum.[23]

Previous detection of RNA was accomplished by traditional molecular biological techniques such as Northern analysis or protection assays. With the advent of RT-PCR, the ability to detect rare species of mRNA or viral RNA was enhanced because of the amplification effect of these rare species. A lengthy description of the procedure is beyond the scope of this article, and the reader is referred to a standard manual on PCR techniques.[31]

Briefly, cellular or virion-associated RNA from fluid or tissue is obtained by some extraction[12] or lysis method. The resulting RNA is

From the Division of Infectious Diseases, Department of Veterans Affairs Medical Center, Palo Alto; and the Department of Medicine, Stanford University, Stanford, California

reverse transcribed via a reverse transcriptase enzyme to cDNA. This DNA can then can be amplified with a primer pair specific for the sequence of interest. By varying PCR cycle number, one can exploit RT-PCR to increase sensitivity by increased cycle numbers. This may affect specificity or result in false-positive reactions because of miss-priming events. Lower cycle numbers can result in quantitative results but may sacrifice sensitivity leading to false-negative results. PCR product or amplicon can be detected or quantitated in a variety of ways, such as ethidium bromide gel electrophoresis, high-pressure liquid chromatography, capillary electrophoresis, direct incorporation of radioactive isotope–labeled dNTPs into amplicon, or hybridization techniques using solid phase membrane or liquid. This can subsequently be followed by electrophoresis or ELISA on microplates utilizing isotopically or nonisotopically labeled (enzyme or chemiluminescent) probes with specific complimentary sequences to the amplicon.

The RT-PCR reaction requires a priming event to initiate RT. This can be accomplished by the use of random hexamers, oligo dT, or the downstream PCR primer. Some investigators believe that random hexamers yield better results; however, all of the priming events have been used with approximately equal efficacy.[31] Different reverse transcriptases can be used, such as an avian or a murine leukemia virus reverse transcriptase. Although a rigorous comparison has not been published, the results with either enzyme appear to be equal.[31] Recently, a thermostable reverse transcriptase has been described, which also has DNA polymerase activity.[48] Thus, one enzyme is capable of RT and PCR by varying the active cation from manganese to magnesium in the RT and PCR reactions, respectively. The use of this enzyme has now been applied to quantitation of HIV virion-associated RNA in plasma.[45]

OTHER TECHNOLOGY

In addition to RT-PCR, other amplification technologies have been described to detect or quantitate HIV genetic sequences. The 3SR reaction, originally described by Guatelli et al,[18] is an isothermal three-enzyme system that can also amplify RNA sequences. This system incorporates the use of three enzymes, namely, reverse transcriptase, RNAase H, and T7 RNA polymerase. Briefly, double-stranded cDNA is synthesized from RNA by reverse transcriptase. The template RNA is degraded by RNAase H. Transcription of an antisense RNA from the transcription-competent cDNA by T7 RNA polymerase results in an RNA copy that continues the cycle. The 3SR reaction has been used to detect RNA species from HIV-infected CEM cells with good results.

A transcription-based amplification system (TAS) also has been described. The system described by Kwoh et al[35] involves the synthesis of a cDNA from an RNA target by reverse transcriptase from a primer that incorporates a binding sequence for a T7 polymerase and a target complimentary sequence. Subsequent incubation of the double-stranded cDNA with T7 RNA polymerase results in the synthesis of

multiple RNA transcripts from the double-stranded DNA template. The cycle can be repeated multiple times, resulting in a tremendous amplification of the original RNA sequence. A recent study by Davis et al[14] compared the use of TAS versus RT-PCR for the detection of HIV RNA and found them to be almost equivalent in detection ability. A recent study by Bush et al[9] compared the use of 3SR technology with plasma culture for the detection of RNA in HIV-infected plasma of infants. In the 19 pediatric patients sampled, there was agreement between both assays in 14 of 19 samples. Of the five samples that were discordant, four were positive by 3SR and negative by culture, which implied an enhanced sensitivity with the use of 3SR when compared with culture.

Another new assay describes the use of branch DNA signal amplification in which HIV RNA in plasma is bound to a solid phase in a microplate well. A DNA probe specific for a *pol* gene sequence is hybridized to the RNA, forming a RNA/DNA hybrid. The branch nature of the DNA allows for the attachment of alkaline phosphatase molecules and a subsequent chemiluminescent reaction, which is quantitative in nature.[51] The ligase chain reaction (LCR) is being applied for amplification and single nucleotide discrimination in DNA sequences. Thermostable DNA ligases have been found that attach perfectly complimentary adjacent oligonucleotides to a target DNA sequence. If a base mismatch appears, there will be no effective ligation, and, therefore, no amplified product will be obtained.[6] Although effectively described for specific DNA amplification, it is easy to envision the application of this technology with prior RT and subsequent LCR, possibly for analysis of quasispecies or nucleoside-resistant strains of HIV.

ASSAY PRECISION AND SAMPLE HANDLING ISSUES

Of critical importance in the application of RT-PCR assays for monitoring HIV-infected patients are issues that revolve around the reproducibility and the precision of the assay and the stability of RNA in clinical samples. The majority of this work has been described in the assays used to detect and quantitate virion-associated RNA in the plasma of HIV-infected patients. Aoki-Sei et al[1] have described various assay conditions that might affect RNA-PCR. A volume relationship was found in patient samples whereby an increase in plasma volume resulted in an increase in HIV particle detection after ultracentrifugation. Plasma had an eightfold higher particle count than the equivalent serum sample from patients with high particle counts ($\geq 10^5$/mL). There was no difference in detectable signal with a cycle of freeze-thaw. Interexperimental and intraexperimental data suggested that variability of the test on a particular sample was within one-third of an order of magnitude. We have also shown that there is good correlation between samples from the same individual which are extracted, reverse transcribed, and amplified on the same day. However, when samples are run on separate days, there is less concordance. Multiple replicates of the same

PCR reaction yielded less than 10% variation in absorbance in an RT-PCR ELISA format.[25] This has been further characterized by Semple et al[59] who found that assay reproducibility studies on 24 plasma samples revealed a mean difference of 3.4% between duplicate pairs. Intra-assay reproducibility for moderately viremic samples varied by 9% but was increased in the low viremic samples up to 15% of the mean value. As expected, the intra-assay variance on different days was greater; the difference between mean values was 25% for moderate viremic values and 79% for low viremic values.

A recently completed multicenter study attempted to evaluate several assays that quantitate plasma HIV RNA. An identical blinded panel of nine samples was provided to several laboratories. All of the laboratories correctly identified the negative sample, but the interlaboratory variation for positive samples was a 1- to 2-log difference in RNA concentration.[71] Lu showed that serum from HIV-infected patients stored for 6 to 12 months yielded stable levels of HIV RNA by RT-PCR. Lu also showed that fresh serum samples could be held up to 4 days at 37°C and have stable RNA copy numbers.[40] The precise sample handling procedure used for these samples was not discussed, and benchtop results utilizing whole blood have yielded a different result when analyzed by our group.[44] When whole citrated blood was held at room temperature for up to 72 hours before plasma separation to simulate sample transport conditions, a precipitous fall off of as much as 80% of the RNA copy number in plasma was found. Thus, if serum or plasma is immediately separated from cellular constituents, RNA can remain stable for up to several days at room temperature, and the results obtained from shipped material will be the same as those from material processed within a 2- to 4-hour time frame.

Collection in specific anticoagulants may have an effect on RT-PCR. We reported that samples collected in heparin could affect results obtained from DNA PCR and RT-PCR of RNA associated in cells and of virion-associated RNA in plasma.[26] Although DNA and mRNA from PBMCs were clearly detectable in samples collected in the presence of heparin, there was a significant quantitative difference in terms of PCR product obtained in comparison with EDTA or citrate samples. This was magnified when plasma samples were tested for the presence of HIV RNA by RT-PCR; plasma samples collected in heparin yielded no detectable signal. Thus, citrate or serum has been touted as the appropriate material for collection of HIV RNA in plasma. Heparinase can reverse the effect seen in samples amplified in the presence of heparin. This has been confirmed recently by Imai et al[28] who demonstrated the recovery of HIV RNA from heparinized plasma utilizing heparinase. Alternative strategies for the collection of plasma HIV RNA could include ultracentrifugation to pellet HIV virions, with subsequent removal of the supernatant containing heparin, or particle capture via latex beads which are coated with IgG monoclonal antibodies to gp41 or gp120.[21] Coombs et al[13] reported plasma RNA stability and handling factors using immunocapture cDNA PCR. The signal appeared to be higher in plasma compared with serum in three of seven subjects. There did

not appear to be a difference in signal from plasma collected in the presence of heparin or citrate nor when plasma (not whole blood) was kept at ambient temperature for 2 and 6 days and freeze-thawed up to nine times. Plasma supernatants were not tested for HIV RNA content after particle capture. Thus, neutralized or immunecomplexed virus might not be detected by this method. Currently, it is probably prudent when quantification of HIV RNA in plasma is contemplated that citrate-containing plasma be used. Primer design may allow selective amplification of HIV RNA in the presence of contaminating DNA whereby DNAase treatment and, thus, an additional step may no longer be necessary.[33]

QUANTIFICATION

Although detection of PCR products by RT-PCR in HIV disease is important, particularly in the diagnosis of pediatric infection, the desire for quantification to measure disease progression or antiretroviral effect has become much more important. Several methods to quantitate PCR product have been reported recently. An external RNA control was described by our group, which utilizes a *gag* gene RNA standard constructed from a plasmid with a T7 promoter and *gag* gene insert. This external control is utilized in a separate reaction series that employs the same *gag* gene primer pair used for the patient sample RT-PCR reaction.[25] When coupled with a nonisotopic ELISA-based microplate format, a log linear relationship between absorbance and copy number is seen over a 3-log range. The advantage of such a system revolves around the fact that one patient sample in duplicate or triplicate can be assayed with an entire external standard curve. This would not control for individual tube variation in the RT-PCR reaction.

Aoki-Sei et al[1] utilized an external standard curve with an HIV_{IIIB} virus stock as the standard curve. Rabbit globin mRNA was added to each plasma sample as an internal control to check for RT efficiency using an additional primer pair. Yerly et al[72] employed a semiquantitative approach by absorbing viral particles in plasma to beads, performing RT and then utilizing a LAV-8E5 DNA dilution series for semiquantitation.

Other groups have described the concept of an internal competitive template for HIV quantitation. Originally described by Wang and Mark,[68] the strategy here is to co-amplify a competitive template with the target template, either using a mutant with a new restriction site, which can be distinguished from the target by restriction enzyme digestion and size fragmentation, or by creation of a deletion mutant, which utilizes the same primer pair sequence as the target but can be resolved from the target on the principle of size with electrophoresis separation. Stieger et al[64] were successful in creating an internal HIV *gag* gene standard with a unique Bgl II restriction site. Both target and competitor were amplified with the same primer pair. The PCR product was digested with Bgl II, hybridized to a radioactive probe, and resolved by size using gel electrophoresis.

Piatak et al[53] have recently described quantitative competitive PCR. They constructed a competitive template in the *gag* region with an 80-base pair deletion and from which a dilution series of known copy number of the competitive template can be co-amplified with a target sequence of unknown copy number. Product is quantitated by ultraviolet fluorescence on ethidium bromide–stained gels. This method was applied to clinical samples from patients with acute infection or to measure a response to antiretroviral therapy.[54]

Nucleic acid sequence–based amplification allows direct isothermal amplification of patient plasma HIV RNA with an internal RNA standard. The standard is constructed in such a fashion as to have equal amplification efficiency. After amplification, specific radioactive probes for wild-type and the RNA internal standard are hybridized to the products and counted.[67] The major limiting factor in such a scheme is the multiple dilutions required for each sample of RNA.

Although it is desirable to have an internal competitive sequence, it may not be practical for large-scale testing in clinical trials in which multiple patients and multiple time points are contemplated. The ability for high throughput of patient sample processing may be difficult unless automated systems are developed and applied. An additional reason for having an internal control rather than an external control is the fact that minor differences in RT or amplification efficiency could result in wide differences in quantitative results. If both the target template and the internal control are subjected to the same reaction conditions in RT-PCR, some of these variabilities and inefficiencies will be controlled for. The one aspect of RT-PCR that is not controlled for in such competitive assays is the nucleic acid extraction step. Preliminary data from Menzo et al[41] demonstrated that, by extracting a known number of HIV RNA copies, a 36% mean loss was observed after extraction, implying that one third of potential clinical sample copy number could be lost on the extraction procedure alone. Thus, the ultimate quantitative assay will probably require that a control template be placed in raw plasma before extraction to control for this variable.

CLINICAL APPLICATION

Although most studies have looked at HIV RNA in PBMCs and plasma, other body fluids also have been studied. Spector et al[65] used RT-PCR to detect virion-associated RNA in the cerebral spinal fluid of 25 seropositive patients. RNA was detected in 11 of 11 samples that were culture-positive and in 9 of 14 samples that were culture-negative, implying a much greater sensitivity using PCR to detect HIV RNA in spinal fluid. Also of note, 6 of 10 samples that were DNA PCR–negative were RT-PCR–positive. There was no apparent correlation with neurologic disease, and zidovudine therapy did not alter the culturability of virus or the presence of HIV RNA in spinal fluid. Yolken et al[73] studied fecal shedding of HIV in pediatric patients. Of 19 patients, 9 presented with diarrhea, and, of those, 5 of 9 were positive for HIV in the stool as

detected by RT-PCR. Of the 10 patients without diarrhea, only 1 of 10 was positive for HIV in the stool, and although only six patients were analyzable, there was a quantitative difference in the amount of HIV RNA copies detected in those patients with diarrhea in comparison with the one patient without diarrhea.

Mermin et al[42] and Hamed et al[19] studied the ability to detect virion-associated RNA in cell-free seminal fluid. Fifteen of 23 HIV-infected subjects (65%) and 30 of 52 (58%), respectively, were positive for HIV RNA by RT-PCR utilizing a *gag* gene primer pair. The presence of RNA in seminal fluid also correlated with the presence of serum p24, lower CD4 counts, and the presence of HIV DNA in nonspermatozoa mononuclear cells in semen.

Gianetti et al[17] recently reported on the presence of spliced *tat* mRNA via RT-PCR in epidermal Langerhans cells. Of nine patients who were in Centers for Disease Control group IV, six of nine had mRNA present in Langerhans cells, whereas zero of nine of the Langerhans cells–depleted epidermal cell fractions contained any HIV RNA.

Pantaleo et al[52] have recently reported on the relationship between peripheral blood and lymph node tissue with respect to viral load. Twelve patients had both PBMCs and lymph node mononuclear cells (LNMC) analyzed for the presence of *gag* gene and *tat/rev* mRNA expression. Semiquantitatively in the early stage of disease, expression of *gag* and *tat/rev* was barely detectable in PBMCs but was found to be at a high level in LNMCs. In late-stage patients there was an increase in expression in PBMCs but still less expression than that found in LNMCs, and both adenoids and tonsillar tissue contained great quantities of mRNA expression. These findings may have important implications in terms of HIV pathogenesis and future clinical trials.

Wormser et al[70] studied eccrine sweat for the presence of HIV RNA and proviral DNA. No HIV viral DNA or RNA was detected in 40 sweat samples tested. Although 15 patients were receiving antiretroviral therapy regimens, 24 patients were not, which implies that the finding of no HIV RNA in sweat is probably a real phenomenon.

Kaneshima et al[29] utilized a SCID mouse model to measure the effect of zidovudine on plasma RNA levels. Using an RT-PCR format, they were able to show that virion-associated HIV RNA was detectable in 30% of infected mice by day 7 and in more than 95% of infected mice by day 14 and was tissue culture, infectious dose–dependent. A zidovudine effect also could be demonstrated on plasma RNA levels in this animal model.

Other important applications have included the study of in vitro transcription events to define temporal kinetics of mRNA expression, novel mRNAs, and in vitro response to therapy. Klotman et al[32] and Stevenson et al[63] have studied the kinetics of expression of HIV RNA species utilizing quantitative RT-PCR. Employing cell lines, Seshamma et al[61] used RT-PCR to quantitate the relationship of intracellular multispliced and unspliced RNA in both stimulated and unstimulated cells. Arrigo et al[2] used RT-PCR in vitro to demonstrate regulation of specific *tat/rev* in RNA. We have described the use of RT-PCR techniques to

monitor the kinetics of acutely infected PBMCs for viral replication, antiviral activity, and viral drug resistance.[69] Thus, specific questions regarding both pathogenesis and antiretroviral activity can easily be studied using RT-PCR techniques.

CELLULAR HIV RNA REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

Hart et al[20] evaluated 21 patients using a spliced *tat* mRNA sequence via RT-PCR and found that 13 of 21 (62%) had positive RNA signal of varying intensity. Also of note, five of six patients who were p24-antigenemic were also positive for *tat* mRNA. Interestingly, four of the subjects were receiving zidovudine and still had positive signal for *tat* mRNA. Thus, in this early qualitative study utilizing a single time point, some distinction could be made as to which patients had transcriptionally active virus. Patients who were negative for RNA in this study all had high CD4 counts greater than 200 cells/mm^3.

Bagnarelli et al[5] assayed 37 patients for both proviral DNA and cellular RNA using a *gag* gene primer pair. This was a detection study only using 35 cycles of amplification, and no information was given as to whether patients were receiving antiretroviral therapy. Of the 23 patients who had CD4 counts greater than 400 cells/mm^3, all were positive for proviral DNA, and 12 of 23 were positive for *gag* gene RNA by RT-PCR. Two of 23 patients were p24-antigenemic. Of 14 patients with less than 400 CD4 cells/mm^3, all were positive for proviral DNA, and 11 of 14 were positive for *gag* gene RNA by RT-PCR; 9 of 14 patients were p24-antigenemic.

Schnittman et al[58] studied 49 patients in all stages of disease, 23 of whom were receiving antiretroviral therapy. The *gag* gene, envelope, major splice junction, and *tat/rev* mRNAs were investigated. A tyrosine kinase mRNA control was included. Results showed that 84% of subjects had expression of at least one mRNA species, and that the spliced *tat/rev* mRNA was the most common, found in 29 of 49 patients. There did not appear to be any association between the stage of disease and the pattern of mRNA expression, and *gag* gene mRNA was found more frequently in those subjects with a lower CD4 percentage. It was hypothesized that, in this study, antiretroviral therapy could have altered the pattern of mRNA expression in these patients.

Michael et al[43] studied 31 subjects in regards to their total and genomic intracellular RNA using a beta-actin gene mRNA as a control. Both total cellular and genomic HIV RNA demonstrated a 100-fold increase in copy number with decreased CD4 count. However, RNA could be demonstrated at all stages of disease. Viral RNA was ultimately detected in only 21 of 31 patients. An increase in the *gag* RNA to DNA ratio was demonstrated in patients of all stages, but the ratio was greatest in those patients with decreased CD4 counts. In terms of disease progression, there was a shift toward genomic RNA versus spliced mRNA.

Clementi et al[11] studied 45 patients, 8 of whom were also hepatitis B–positive. A total of 34 of 45 patients (75%) had evidence of HIV *gag* gene transcripts in PBMCs, 16 of 19 with CD4 counts less than 400 cells/mm^3, and 18 of 26 with CD4 counts greater than 400 cells/mm^3. Of the eight subjects who were hepatitis B and HIV-positive, all eight demonstrated HIV provirus and hepatitis B DNA by PCR, and, in addition, eight of eight had HIV RNA *gag* gene transcripts present. Hepatitis B transcripts in the S and C regions were positive in three of eight and six of eight patients, respectively, thus, demonstrating active transcription of both HIV and HBV simultaneously.

Bagnarelli et al[3] studied 33 patients for virion-associated plasma RNA, PBMC proviral DNA, and PBMC *gag* gene mRNA. All three markers showed a trend toward a quantitative increase with disease progression, and when the markers were analyzed together, a significant correlation was seen between plasma RNA, proviral DNA, and active *gag* gene expression.

Seshamma et al[62] studied 52 subjects who were not receiving antiretroviral therapy. By RT-PCR high levels of multiply spliced RNA and low levels of unspliced RNA were demonstrated in asymptomatic subjects. In patients with ARC and AIDS, the level of unspliced RNA was much higher. In fact, 50% of asymptomatic subjects had no unspliced RNA. This led the investigators to conclude that some asymptomatic patients may have a large reservoir of latently infected cells that are blocked at an early state of infection.

All of the studies described previously have presented data only from a single time point. It is difficult to determine what biologically significant changes may be seen within a patient when no longitudinal data are described. Furtado et al[16] recently studied 10 patients before and after the initiation of zidovudine therapy to detect PBMC proviral DNA and *tat* mRNA expression. Eight of 10 patients showed a decrease in *tat* mRNA and proviral DNA levels 4 to 20 weeks after the initiation of therapy. However, in 2 of 10 patients, there was an increase in both *tat* RNA and proviral DNA. No information was given as to why this could have occurred in these patients. Tetali et al[66] found that if PBMCs were stimulated first before RT-PCR, this resulted in increased sensitivity. Eleven asymptomatic subjects had PBMCs collected at time zero and 36 hours after incubation with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA). Using a *gag* gene sequence and quantitative RT-PCR, a statistically significant increase in *gag* gene mRNA was demonstrated after stimulation. No other mRNA species were studied. This may prove to be a useful method for identifying HIV mRNA in asymptomatic patients or for studying ex vivo activation.

PLASMA HIV RNA REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

Although infectious plasma HIV viremia is present only in certain patients, investigators have explored whether PCR techniques could

increase the sensitivity of detection of cell-free virus. Hewlett et al[23] reported on four HIV-infected subjects who were studied retrospectively after acute seroconversion. A semiquantitative presence of both HIV RNA and DNA was described in the serum of these patients. This was one of the first reports of detectable HIV RNA in serum. These investigators subsequently studied HIV-infected hemophiliacs and their sexual partners and children for the presence of HIV DNA in serum. Of 27 partners, 5 were seropositive and PCR-positive, 11 were negative for both, and surprisingly, 11 were seronegative and PCR-positive. This study also used HLA primers on all samples, which revealed that all of the samples tested were positive for HLA, implying that amplifiable DNA in serum had come from PBMCs. Also reported were cases of PCR positivity in seronegative offspring of PCR positive–seronegative mothers.[22]

Busch et al[8] further evaluated this method in a blinded fashion. They found a 25.5% sensitivity and 18.5% false-positive rate for the detection of HIV DNA in serum. They concluded that this was a poorly reproducible marker of HIV infection. We have used a formal RNA extraction method on serum and have found no detectable signal without prior RT.[25] Bruisten et al[7] further confirmed the lack of HIV DNA in plasma but reported that 56 of 70 (80%) plasma samples contained HIV RNA by RT-PCR. In a platelet-rich fraction of plasma, 29 of 63 (46%) samples revealed HIV RNA by RT-PCR. This implies a compartmental model whereby HIV RNA contained in plasma may be truly cell-free or platelet-associated.

We and others have reported that patients without demonstrable infectious virus may have HIV RNA present in plasma or serum.[25,54] In addition, several studies have demonstrated a correlation with stage of disease, p24 antigenemia, or CD4 counts.[1,15,25,39,54,59,60,72] Some have reported the presence of HIV RNA in plasma during the time of acute infection.[54] In a study by Henrard et al[21], one patient demonstrated RNA in plasma 5 to 8 months before the presence of antibody, and, in another patient, RNA in plasma was detected 2 days before the presence of p24 antigen. In another case report de Saussure et al[56] described a patient who demonstrated HIV RNA in plasma 18 days before p24 antigenemia and 36 days before HIV antibody formation.

Other relevant studies describe the ability to co-amplify sequences of both hepatitis C virus and HIV from the sera of patients with dual infection. Nedjar et al[49] reported that hepatitis C virus was detected in the sera of all 13 subjects and HIV RNA in 11 of 13, thus, demonstrating the feasibility of such a strategy. Other infections may likewise affect RNA levels in plasma. Semple et al[60] described an acute 10-fold rise in plasma HIV RNA with the onset of herpes simplex infection, despite uninterrupted zidovudine therapy. This suggests that other factors such as concomitant herpetic infections may be involved in active HIV replication and perhaps impact on plasma HIV RNA levels.

In studies evaluating the ability to detect RNA in plasma during antiretroviral therapy, Ottman et al[50] were successful in detecting HIV RNA in plasma in 95% of subjects evaluated, including in 24 of 25

subjects receiving zidovudine. Bagnarelli et al[4] examined 27 subjects, 5 of whom were receiving azidothymidine therapy; four of five had detectable plasma HIV RNA, and three of these four had cellular HIV mRNA present as well. All of these patients had CD4 counts below 200 cells/mm^3, three of five were p24-antigenemic, and the methodological approach involved ultracentrifugation to sediment virus, enhancing virion-associated HIV RNA recovery. In addition, 40 and 35 cycles of amplification after reverse transcription were performed by Ottman and Bagnarelli, respectively. This method would increase the sensitivity of such an assay to detect HIV RNA, but the ability to show quantitative changes unless a limiting dilution were performed would be lost. Scadden et al[57] reported on 11 patients, all with CD4 counts less than 200 cells/mm^3. Four of eleven patients were not currently receiving antiretroviral therapy, and two of these four had therapy withdrawn within 4 weeks of this study. Mean copy number for the subjects not receiving therapy was significantly higher than that for patients receiving therapy.

In studies of therapeutic response, Karpas et al[30] reported that serum HIV RNA and DNA levels fell to undetectable levels in 10 subjects with advanced HIV disease after 2 months of passive immunoglobulin therapy, suggesting an immune therapy–based response in circulating HIV RNA load. However, another study of the quantification of HIV proviral DNA in five patients with AIDS who received immunoglobulin therapy showed no significant decrease in provirus when compared with that in three patients who were receiving placebo.[38] In the latter study, HIV plasma RNA was not measured.

Several studies have reported an acute decrease in plasma HIV RNA after the initiation of zidovudine therapy.[1,27,39,54,59,60] Semple et al[59] and Piatak et al[54] reported rapid decreases in HIV RNA in five and four patients, respectively, as soon as 1 week after the initiation of zidovudine. Piatak et al[54] also reported on three patients who discontinued zidovudine therapy after 6 weeks and who experienced a rebound in plasma RNA after 1 week off therapy. Aoki-Sei et al[1] reported an acute decrease in plasma HIV RNA in 10 of 11 subjects who received dideoxyinosine for 8 weeks or greater. A decrease was also seen in seven patients who had received dideoxyinosine for 46 to 70 weeks.[1] Recently, we evaluated 72 patients in a cross-sectional study of HIV disease to determine the impact of zidovudine therapy on plasma HIV RNA copy number. Thirty-nine subjects who were not currently receiving therapy had a mean copy number of 690 ± 360 in comparison with 33 subjects currently receiving zidovudine who had a mean copy number of 134 ± 219/200 μL ($P < 0.05$). In addition, 27 subjects were evaluated before and 1 month after the initiation of dideoxynucleoside therapy. Plasma HIV RNA copy number decreased from 540 ± 175 to 77 ± 35 after therapy ($P < 0.05$).[27]

Data also have been reported in regards to the long-term effect of antiretroviral therapy on plasma RNA levels. We recently reported on five drug-naive, asymptomatic subjects who initiated combination therapy with dideoxyinosine and zidovudine. Plasma RNA levels showed a dramatic fall at 1 month, with continued suppression after 12 months of

combination therapy.[24] Proviral DNA levels demonstrated a transient fall at 3 months. Lu and Andrieu[39] evaluated 32 asymptomatic subjects given zidovudine for 6 months. When PBMC coculture and plasma RNA were studied, three groups emerged: (1) responders, (2) partial responders, and (3) nonresponders with respect to these two markers. Earlier decreases in PBMC viral load correlated with later declines in plasma HIV RNA levels. There was no information given regarding this lack of response, but it was hypothesized that it could be related to the development of zidovudine-resistant strains. Kozal et al[34] were able to detect genotypically zidovudine-resistant strains of HIV in plasma using an RT-PCR nested strategy originally described by Larder et al[37] for proviral DNA.

Antiviral response is also being studied in other body fluids. Hamed et al[19] reported on six subjects who had initiated zidovudine therapy. Two of six demonstrated an acute decrease in RNA in seminal fluid after 2 to 4 weeks of therapy. Therefore, it would seem that HIV RNA levels in plasma or other body fluids as detected by RT-PCR may serve as a useful marker of viral load and antiretroviral response. Further longitudinal studies will be necessary to compare and contrast the clinical utility of quantitating cellular HIV RNA or plasma virion-associated RNA.

References

1. Aoki-Sei S, Yarchoan R, Kageyama S, et al: Plasma HIV-1 viremia in HIV-1 infected individuals assessed by polymerase chain reaction. AIDS Res Hum Retroviruses 8:1263–1270, 1992
2. Arrigo SJ, Weitsman S, Rosenblatt JD, et al: Analysis of *rev* gene function on human immunodeficiency virus type 1 replication in lymphoid cells by using a quantitative polymerase chain reaction method. J Virol 63:4875–4881, 1989
3. Bagnarelli P, Menzo S, Valenza A, et al: Molecular profile of human immunodeficiency virus type 1 infection in symptomless patients and in patients with AIDS. J Virol 66:7328–7335, 1992
4. Bagnarelli P, Menzo S, Manzin A, et al: Detection of human immunodeficiency virus type 1 genomic RNA in plasma samples by reverse transcriptase polymerase chain reaction. J Med Virol 34:89–95, 1991
5. Bagnarelli P, Menzo S, Manzin A, et al: Detection of human immunodeficiency virus type 1 transcripts in peripheral blood lymphocytes by the polymerase chain reaction. J Virol Methods 32:31–39, 1991
6. Barany F: The ligase chain reaction in a PCR world. PCR Methods Appl 1:5–16, 1991
7. Bruisten S, Van Gemen B, Koppelman M, et al: Detection of HIV-1 distribution in different blood fractions by two nucleic acid amplification assays. AIDS Res Hum Retroviruses 9:259–265, 1993
8. Busch MP, Henrard DR, Hewlett IK, et al: Poor sensitivity, specificity, and reproducibility of detection of HIV-1 DNA in serum by polymerase chain reaction. J AIDS 5:872–877, 1992
9. Bush CE, Donovan RM, Peterson WR, et al: Detection of human immunodeficiency virus type 1 RNA in plasma samples from high-risk pediatric patients by using the self-sustained sequence replication reaction. J Clin Microbiol 30:281–286, 1992
10. Byrne BC, Li JJ, Sninsky J, et al: Detection of HIV-1 RNA sequences by in vitro DNA amplification. Nucleic Acids Res 16:4165, 1988
11. Clementi M, Manzin A, Bagnarelli P, et al: Human immunodeficiency virus type 1 and hepatitis B virus transcription in peripheral blood lymphocytes from co-infected subjects. Arch Virol 126:1–9, 1992

12. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid-guanadinium-thiocynate-phenol-chloroform extract. Ann Biochem 162:156–159, 1987
13. Coombs RW, Henrard DR, Mehaffey WF, et al: Cell-free plasma human immunodeficiency virus type 1 titer assessed by culture and immunocapture-reverse transcription-polymerase chain reaction. J Clin Microbiol 31:1980–1986, 1993
14. Davis GR, Blumeyer K, DiMichele LJ, et al: Detection of human immunodeficiency virus type 1 in AIDS patients using amplification-mediated hybridization analyses: Reproducibility and quantitative limitations. J Infect Dis 162:13–20, 1990
15. Escaich S, Ritter J, Rougier P, et al: Plasma viraemia as a marker of viral replication in HIV-infected individuals. AIDS 5:1189–1194, 1991
16. Furtado MR, Murphy R, Wolinsky SM: Quantification of human immunodeficiency virus type 1 tat mRNA as a marker for assessing the efficacy of antiretroviral therapy. J Infect Dis 167:213–216, 1993
17. Gianetti A, Zambruno G, Cimarelli A, et al: Direct detection of HIV-1 RNA in epidermal Langerhans cells of HIV-infected patients. J AIDS 6:329–333, 1993
18. Guatelli JC, Whitfield KM, Kwoh DY, et al: Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci USA 87:1874–1878, 1990
19. Hamed K, Winters M, Holodniy M, et al: Detection of human immunodeficiency virus type 1 in semen: Effects of disease stage and nucleoside therapy. J Infect Dis 167:798–802, 1993
20. Hart C, Schochetman G, Spira T, et al: Direct detection of HIV RNA expression in seropositive subjects. Lancet 2:596–599, 1988
21. Henrard DR, Mehaffey WF, Allain J: A sensitive viral capture assay for detection of plasma viremia in HIV-infected individuals. AIDS Res Human Retroviruses 8:47–52, 1992
22. Hewlett IK, Laurian Y, Epstein J, et al: Assessment by gene amplification and serological markers of transmission of HIV-1 from hemophiliacs to their sexual partners and secondarily to their children. J AIDS 3:714–720, 1990
23. Hewlett IK, Gregg RA, Ou CY, et al: Detection in plasma of HIV-1 specific DNA and RNA by polymerase chain reaction before and after seroconversion. J Clin Immunoassay 11:161–164, 1988
24. Holodniy M, Katezenstein D, Winters MA, et al: Measurement of HIV virus load and genotypic resistance by gene amplification in asymptomatic subjects treated with combination therapy. J AIDS 6:366–369, 1993
25. Holodniy M, Katzenstein DA, Sengupta S, et al: Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. J Infect Dis 163:862–866, 1991
26. Holodniy M, Kim S, Katzenstein D, et al: Inhibition of human immunodeficiency virus gene amplification by heparin. J Clin Microbiol 29:676–679, 1991
27. Holodniy M, Katzenstein DA, Israelski DM, et al: Reduction in plasma human immunodeficiency virus ribonucleic acid after dideoxynucleoside therapy as determined by the polymerase chain reaction. J Clin Invest 88:1755–1759, 1991
28. Imai H, Yamada O, Morita S, et al: Detection of HIV-1 RNA in heparinized plasma of HIV-1 seropositive individuals. J Virol Methods 36:181–184, 1992
29. Kaneshima H, Shih C, Namikawa R, et al: Human immunodeficiency virus infection of human lymph nodes in the SCID-hu mouse. Proc Natl Acad Sci USA 88:4523–4527, 1991
30. Karpas A, Hewlett IK, Hill F, et al: Polymerase chain reaction evidence for human immunodeficiency virus 1 neutralization by passive immunization in patients with AIDS and AIDS-related complex. Proc Natl Acad Sci USA 87:7613–7617, 1990
31. Kawasaki ES: Amplification of RNA. *In* Innis MA, Gelfand DH, Sninsky JJ, et al (eds): PCR Protocols: A Guide to Methods and Applications. San Diego, Academic, 1990, pp 21–69
32. Klotman M, Kin S, Buchbinder A, et al: Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes. Proc Natl Acad Sci USA 88:5011–5015, 1991
33. Kollmann TR, Zhuang X, Rubinstein A, et al: Design of polymerase chain reaction primers for the selective amplification of HIV-1 RNA in the presence of HIV-1 DNA. AIDS 6:547–552, 1992

34. Kozal MJ, Safer RW, Winters MA, et al: A mutation in human immunodeficiency virus reverse transcriptase and decline in CD4 lymphocyte numbers in long-term zidovudine recipients. J Infect Dis 167:526–532, 1993
35. Kwoh DY, Davis GR, Whitfield KM, et al: Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci USA 86:1173–1177, 1989
36. Kwok S, Mack DH, Mullis KB, et al: Identification of human immunodeficiency virus sequences by using in vitro enzymatic amplification and oligomer cleavage detection. J Virol 61:1690–1694, 1987
37. Larder BA, Kellam P, Kemp SD: Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. AIDS 5:137–144, 1991
38. Lefere JJ, Mariatee M, Poste Y, et al: Quantitation of viral copies through PCR in AIDS patients transfused with plasma rich in anti-HIV antibodies [abstract W.B. 2132]. Presented at the VII International Conference on AIDS, Florence, Italy, 1991
39. Lu W, Andrieu J: Early identification of human immunodeficiency virus-infected asymptomatic subjects susceptible to zidovudine by quantitative viral coculture and reverse transcription-linked polymerase chain reaction. J Infect Dis 167:1014–1020, 1993
40. Lu W, Andrieu J: Use of the human immunodeficiency virus virion as a universal standard for viral RNA quantitation by reverse transcription-linked polymerase chain reaction. J Infect Dis 167:1498–1499, 1993
41. Menzo S, Bagnarelli P, Giacca M, et al: Absolute quantitation of viremia in human immunodeficiency virus infection by competitive reverse transcription and polymerase chain reaction. J Clin Microbiol 30:1752–1757, 1992
42. Mermin JH, Holodniy M, Katzenstein D, et al: Detection of human immunodeficiency virus DNA and RNA in semen by the polymerase chain reaction. J Infect Dis 164:769–772, 1991
43. Michael NL, Vahey M, Burke DS, et al: Viral DNA and mRNA expression correlate with the stage of human immunodeficiency virus (HIV) type 1 infection in humans: Evidence for viral replication in all stages of HIV disease. J Virol 66:310–316, 1992
44. Mole L, Margolis D, Carroll R, et al: Stability of quantitative HIV plasma culture and RNA and proliferation assays from samples collected in Leucoprep and Vacutainer tubes [abstract PO-B41-2486]. *In* Proceedings of the IX International Conference on AIDS, Berlin, Germany, 1993, p 549
45. Mulder J, McKinney N, Christopherson C, et al: Quantitation of HIV plasma viremia by PCR with Tth DNA polymerase [abstract PO-A32-0777]. *In* Proceedings of the IX International Conference on AIDS, Berlin, Germany, 1993, p 264
46. Mullis K, Faloona F, Scharf S, et al: Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. Cold Spring Harb Symp Quant Biol 51:263–272, 1986
47. Murakawa GJ, Zaia JA, Spallone PA, et al: Direct detection of HIV-1 RNA from AIDS and ARC patient samples. DNA 239:295–297, 1988
48. Myers TW, Gelfand DH: Reverse transcription and DNA amplification by *Thermus thermophilus* DNA polymerase. Biochemistry 30:7661–7666, 1991
49. Nedjar S, Biswas RM, Hewlett IK: Co-amplification of specific sequences of HCV and HIV-1 genomes by using the polymerase chain reaction assay: A potential tool for the simultaneous detection of HCV and HIV-1. J Virol Methods 35:297–304, 1991
50. Ottman M, Innocenti P, Thanadey M, et al: The polymerase chain reaction for the detection of HIV-1 genomic RNA in plasma from infected individuals. J Virol Methods 31:273–284, 1991
51. Pachl C, Lindquist C, Kern D, et al: Quantitation of HIV-1 RNA in plasma using a signal amplification branched DNA (bDNA) assay [abstract]. *In* Proceedings of the IX International Conference on AIDS, Berlin, Germany, 1993, p 40
52. Pantaleo G, Graziosi C, Demarest JF, et al: HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362:355–358, 1993
53. Piatak M, Luk K, Williams B, et al: Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. BioTechniques 14:70–81, 1993
54. Piatak M, Saag MS, Yang LC, et al: High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science 259:1749–1754, 1993
55. Saiki RK, Gelfand DA, Stoffel S, et al: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–493, 1988

56. de S
acid
57. Scad
mun
Dis
58. Schn
mRN
dise
59. Sem
non-
1993
60. Sem
infec
apy.
61. Sesh
poly
1992
62. Sesh
bloo
Proc
63. Stev
the
64. Stie
reac
1991
65. Spec
viru
66. Teta
dete
hanc
1993
67. van
usin
68. Wan
(eds
1990
69. Win
amp
PCR
70. Wor
mun
71. Yen-
quar
Inte
72. Yerl
prov
166:
73. Yolk
nucl
164:

56. de Saussure P, Yerly S, Tullen E, et al: Human immunodeficiency virus type 1 nucleic acids detected before p24 antigenemia in a blood donor. Transfusion 33:164–167, 1993
57. Scadden DT, Wang Z, Groopman JE: Quantitation of plasma human immunodeficiency virus type 1 RNA by competitive polymerase chain reaction. J Infect Dis 165:1119–1123, 1992
58. Schnittman SM, Greenhouse JJ, Lane HC, et al: Frequent detection of HIV-1-specific mRNAs in infected individuals suggests ongoing active viral expression in all stages of disease. AIDS Res Hum Retroviruses 7:361–367, 1991
59. Semple MG, Kay S, Loveday C, et al: HIV-1 plasma viraemia quantification: A non-culture measurement needed for therapeutic trials. J Virol Methods 41:167–180, 1993
60. Semple M, Loveday C, Weller I, et al: Direct measurement of viraemia in patients infected with HIV-1 and its relationship to disease progression and zidovudine therapy. J Med Virol 35:38–45, 1991
61. Seshamma T, Bagasra O, Oakes JW, et al: A quantitative reverse transcriptase-polymerase chain reaction for HIV-1-specific RNA species. J Virol Methods 40:331–346, 1992
62. Seshamma T, Bagasra O, Trono D, et al: Blocked early-stage latency in the peripheral blood cells of certain individuals infected with human immunodeficiency virus type 1. Proc Natl Acad Sci USA 89:10663–10667, 1992
63. Stevenson M, Stanwick TL, Dempsey MP, et al: CA: HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J 9:1551–1560, 1990
64. Stieger M, Demolliere C, Ahlborn-Laake L, et al: Competitive polymerase chain reaction assay for quantitation of HIV-1 DNA and RNA. J Virol Methods 34:149–160, 1991
65. Spector SA, Hsia K, Pratt D, et al: Virologic markers of human immunodeficiency virus type 1 in cerebrospinal fluid. J Infect Dis 168:68–74, 1993
66. Tetali SK, Oyaizu N, Pual M, et al: Human immunodeficiency virus type 1 RNA detection in peripheral blood mononuclear cells by polymerase chain reaction: Enhanced sensitivity after mitogenic stimulation. AIDS Res Hum Retroviruses 9:77–82, 1993
67. van Gemen B, Kievits T, Schukkink R, et al: Quantification of HIV-1 RNA in plasma using NASBA™ during HIV-1 primary infection. J Virol Methods 43:177–188, 1993
68. Wang AE, Mark DF: Quantitative PCR. *In* Innis MA, Gelfand DH, Sninsky JJ, et al (eds): PCR Protocols: A Guide to Methods and Applications. San Diego, Academic, 1990, pp 70–75
69. Winters MA, Holodniy M, Katzenstein DA, et al: Quantitative RNA and DNA gene amplification can rapidly monitor HIV infection and antiviral activity in cell cultures. PCR Methods Appl 1:257–262, 1992
70. Wormser GP, Bittker S, Foreseter G, et al: Absence of infectious human immunodeficiency virus type 1 in "natural" eccrine sweat. J Infect Dis 165:155–158, 1992
71. Yen-Lieberman B, Lin HJ, Jackson B: Multicenter evaluation of methods for the quantitation of plasma HIV-1 RNA [abstract PO-A32-0788]. *In* Proceedings of the IX International Conference on AIDS, Berlin, Germany, 1993, p 266
72. Yerly S, Chamot E, Hirschel B, et al: Quantitation of human immunodeficiency virus provirus and circulating virus: Relationship with immunologic parameters. J Infect Dis 166:269–276, 1992
73. Yolken RH, Li S, Perman J, et al: Persistent diarrhea and fecal shedding of retroviral nucleic acids in children infected with human immunodeficiency virus. J Infect Dis 164:61–66, 1991

Address reprint requests to

Mark Holodniy, MD
Infectious Disease Section(111-ID)
Department of Veterans Affairs Medical Center
3801 Miranda Avenue
Palo Alto, CA 94304