JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1996, p. 404–408 Vol. 34, No. 2
0095-1137/96/$04.00+0

Quantification of Low Levels of Human Immunodeficiency Virus (HIV) Type 1 RNA in P24 Antigen-Negative, Asymptomatic, HIV-Positive Patients by PCR

M. ANGELES MUÑOZ-FERNÁNDEZ,[1]* JOAQUÍN NAVARRO,[1] MIGUEL GARCÍA MONTES,[1] JAIME COSÍN,[2] JOSE MARÍA ZABAY,[1] AND EDUARDO FERNÁNDEZ-CRUZ[1]

Division of Immunology[1] and Infectious Diseases Unit,[2] University General Hospital Gregorio Marañon, Complutense University of Madrid, Madrid, Spain

Received 28 December 1994/Returned for modification 14 February 1995/Accepted 23 August 1995

A nested PCR was used to quantify small numbers of human immunodeficiency virus (HIV) type 1 (HIV-1) RNA particles in the serum specimens of 26 p24 antigen-negative, asymptomatic, HIV-positive patients undergoing antiretroviral therapy. Fifteen patients received zidovudine (ZDV) and alpha interferon, and 11 patients received ZDV monotherapy. After PCR, the amounts of RNA were quantified by comparing the endpoint dilutions of serum samples with a standard curve with known amounts of viral particles. Before the beginning of the antiviral therapy, HIV-1 RNA was detected in 92% of the patients. After treatment, a fall in the number of viral particles was detected in patients receiving combination therapy (mean titers ± standard errors of the means, 3,617 ± 756 pretherapy versus 1,800 ± 845 posttherapy; $P < 0.05$) and in patients receiving monotherapy (3,763 ± 642 pretherapy versus 1,353 ± 394 posttherapy; $P < 0.05$). Our results indicate that PCR with nested primers may be useful for assessing the changes in viremia in HIV-positive patients with low viral load undergoing antiviral therapy.

The natural history of human immunodeficiency virus (HIV) type 1 (HIV-1) infection is characterized by the development of a progressive immune dysfunction, beginning at early stages of the infection (12). In the asymptomatic period of the HIV infection, assays for determining p24 antigenemia and for culturing the virus from peripheral blood mononuclear cells detect only minimal or no viral replication (13, 15). In more-advanced stages of the HIV infection, the viral load measured by plasma culture is significantly associated with clinical manifestations of the infection, immunological abnormalities, and p24 antigenemia (2, 4). However, the use of culture techniques to detect plasma viremia can be limited by three factors: requirement of fast processing, variability in the phytohemagglutinin-stimulated donor cells, and the variability of different clinical isolates of HIV-1 in replication in culture. Furthermore, in most stages of HIV infection, with the exception of AIDS, the standard assays for quantification of p24 antigenemia detect only a minority of HIV-1-infected individuals positive for p24 antigen. In addition, the binding of HIV p24 antigen with its antibody, forming circulating immune complexes, might mask this HIV antigen against detection in serum (6). Recently, the PCR has been widely applied to the detection of HIV proviral DNA and RNA in peripheral blood mononuclear cells from HIV-1-positive patients (8, 10, 16). Furthermore, HIV RNA in plasma has been detected and quantified by RNA extraction followed by reverse transcription and PCR of cDNA (8, 9).

In this study, we have used a nested-PCR method for the detection and quantification of small numbers of HIV RNA particles in the sera of asymptomatic, HIV-positive patients negative for p24 antigenemia. This study shows that the nested PCR is useful in assessing the changes in viremia in HIV-positive patients with low viral load undergoing antiviral therapy.

* Corresponding author. Mailing address: Division of Immunology, University General Hospital Gregorio Marañon, Dr. Esquerdo 46, 28007 Madrid, Spain. Phone: (34-1)-5868460. Fax: (34-1)-5868018.

MATERIALS AND METHODS

Patients. Blood samples were collected by venipuncture in the absence of anticoagulant, and serum samples were isolated within 1 h and stored at −70°C until used. Serum specimens were obtained from 26 HIV-positive, asymptomatic injection drug users (IDUs) (category A according to the Centers for Disease Control and Prevention 1993 revised classification system for HIV infection). Fifteen patients were undergoing combined therapy with zidovudine (ZDV) (250 mg twice daily) plus alpha interferon (3 MU three times per week). Eleven patients were receiving ZDV alone (250 mg twice daily). Fifteen HIV-seronegative, healthy volunteers were used as controls. Informed consent was obtained from the patients, and human experimentation guidelines of the U.S. Department of Health and Human Services were followed in the conduct of this clinical research.

RNA extraction, reverse transcription, and amplification of cDNA. Total RNA was extracted from serum by a previously described method (3). Briefly, 200 μl of serum to which 5 M guanidinium thiocyanate had previously been added was extracted with phenol-chloroform and precipitated with isopropanol. The resulting pellet was then washed in 75% ethanol, dried, and resuspended in 10 μl of diethylpyrocarbonate-treated glass-distilled water. No differences in the sensitivity of the reaction were detected when 1 μg of yeast transfer RNA (Sigma) was used as a carrier. Preparation of samples from HIV-positive patients was done in a biosafety laminar-airflow hood used exclusively for this purpose.

HIV RNA was transcribed to cDNA by using avian myeloblastosis virus reverse transcriptase (Promega) by a previously described method (3).

For the reverse transcription reaction, we used a random hexamer (3) which enabled us to obtain the largest amount of the final amplified product. The PCR assay was performed according to a previously described method (1), with minor modifications. Briefly, we used a set of nested primer pairs (JA9 to JA12) for the *env* region of HIV-1 for amplification of a 341-bp sequence (1). Primers specific for the β-globin gene of human DNA (PC03 and PC04) were routinely used (1). Amplification of HIV cDNA was performed in a two-step reaction, first with a pair of outer primers (JA9 and JA12) and then with a pair of inner primers (JA10 and JA11) (i.e., nested primers). The PCR was performed by adding 10 μl of cDNA to 0.2-ml Microfuge tubes (Perkin-Elmer Corp.) in a total volume of 50 μl. The reaction mixture contained (final concentrations) 10 mM Tris (pH 8.3), 50 mM KCl, and 4.0 mM $MgCl_2$ for JA9 and JA12 and 3.0 mM $MgCl_2$ for JA10 and JA11. The final concentrations of each JA primer and of each deoxynucleoside triphosphate (ATP, CTP, GTP, and TTP) (Pharmacia) in the PCR mixtures were 0.1 and 50 μM, respectively. The *Taq* polymerase was used at a concentration of 1 U/50 μl (Perkin-Elmer). The samples were first denatured for 5 min at 95°C in a Thermal Cycler (Perkin-Elmer GeneAmp PCR system 9600), then cycled 24 times at 94°C for 20 s, annealed at 50°C for 20 s and at 72°C for 30 s

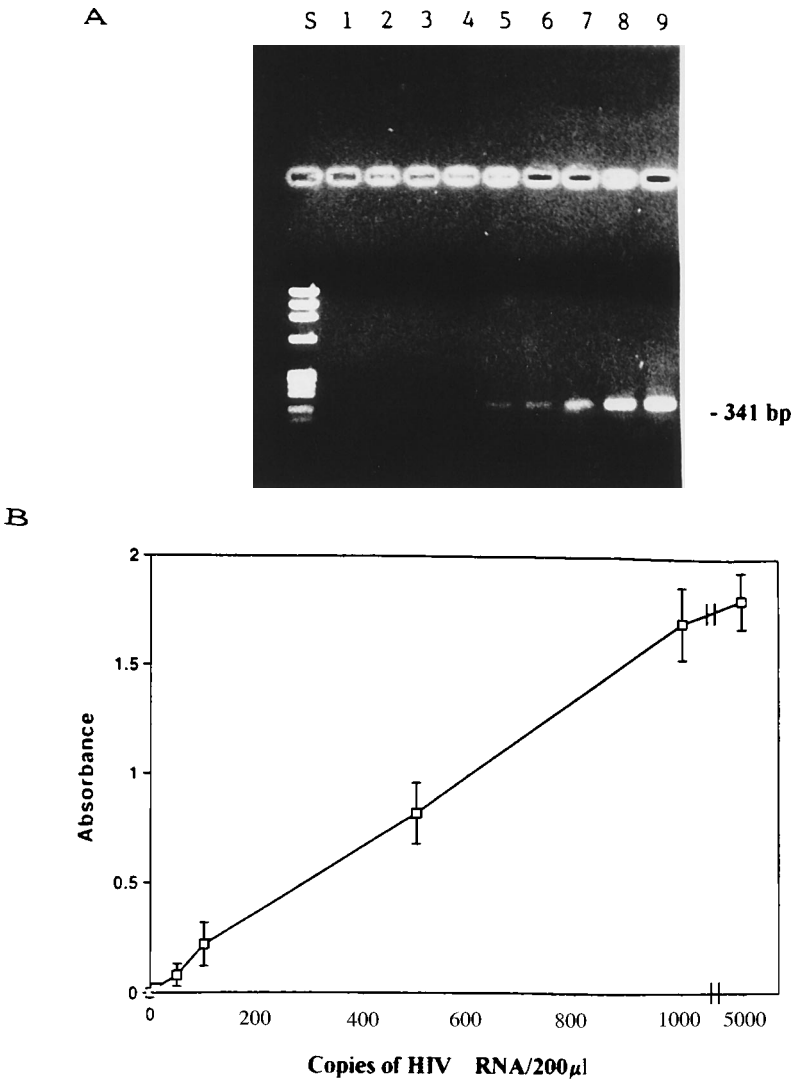

FIG. 1. Quantification of HIV RNA in serum samples. RNA was extracted from serum and reverse transcribed to cDNA. Amplification of HIV cDNA was performed by PCR with nested primers. (A) Electrophoresis of the amplified PCR products on an agarose gel. Lane 1, negative control; lanes 2 to 9, products of nested PCR performed by adding different amounts of a stock of HIV S61 virus (0, 1, 10, 50, 100, 500, 1,000, and 5,000 viral particles, respectively) to 200 μl of HIV-seronegative control serum; lane S, DNA molecular weight markers (ϕX174-*Hin*fI). (B) The amounts of the amplified PCR products corresponding to lanes 2 to 9 in panel A were quantified by densitometric scanning, and the absorbances were plotted against the number of copies of HIV RNA. Shown are the means ± 1 standard deviation (bars) of duplicate samples.

(extending 2 s per cycle), and finally incubated at 72°C for 5 min. After the first PCR, 10% (5 μl) of the product was amplified again for 24 cycles using the corresponding inner primers. The product from the second PCR (10 μl) was detected by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized with short-wavelength UV light. A result was considered positive if a band of any intensity at the correct size was visible on a gel photograph. A Polaroid PN55 negative print was used for densitometric scanning.

Quantification of HIV-1 RNA. The sensitivity and specificity of our nested-PCR method for the detection and quantification of HIV RNA in serum were first assessed in reconstruction experiments. A standard curve was prepared by adding different amounts of a stock of HIV S61 virus (0, 1, 10, 50, 100, 500, 1,000, 5,000, and 10,000 viral particles) to 200 μl of HIV-seronegative control serum. Duplicates of the standard were run at each concentration. The concentration of virus particles in the HIV-1 S61 standard was determined by electron microscopy. HIV RNA in serum was detected and quantified by RNA extraction followed by reverse transcription and PCR of cDNA (Fig. 1A). After PCR, the intensity of the specific band (341 bp) was quantified with a 300A Molecular Dynamic densitometer and the absorbance data were plotted against the number of copies of RNA (Fig. 1B). There was a good linear correlation between the input of viral RNA particles in the serum and the absorbance observed in the range of 10 to 1,000 viral particles per 200 μl (Fig. 1B). For example, for the standard curve (Fig. 1B), the coefficient of linear correlation and the equation of simple regression based on five points (10, 50, 100, 500, and 1,000 viral particles added to 200 μl of HIV-seronegative control serum) were $r = 0.99$ and $y = 0.002x + 0.023$, respectively. This PCR method was able to detect as few as 50 viral particles per ml. The use of greater numbers of cycles of amplification increased the sensitivity of the assay for lower copy numbers but decreased the linear correlation between the viral load and the absorbances (data not shown). Serum samples with more than 5,000 viral particles per ml gave basically the same absorbance (Fig. 1B), regardless of the sample's viral load, indicating that the enzymatic reaction catalyzed by *Taq* polymerase had reached a point of saturation with the substrate.

In all experiments, negative controls containing no template DNA were subjected to the same procedures to detect any possible contamination which might occur during the first and second amplification reactions, although the highest dilutions below a critical limit act as additional negative controls.

To test the reproducibility of the PCR assay, we used seven serum specimens from HIV-positive IDUs with detectable p24 antigenemia and low numbers of $CD4^+$ cells who were undergoing treatment with ZDV and five serum samples from seronegative, healthy controls. The serum samples were subjected to extraction, reverse transcription, and amplification on the same day. Linear regression analysis demonstrated a good correlation of mean absorbance values from separate extractions and reverse transcription ($r = 0.96$, $P < 0.01$; and $r = 0.93$,

TABLE 1. Quantification of HIV-1 RNA in p24 antigen-negative, asymptomatic, HIV-positive patients before and after antiretroviral therapy

Combination therapy group[a]					Monotherapy group[b]				
Patient no.	HIV RNA (copy no./ml)[c]		$CD4^+$ T lymphocytes (cells/mm^3)		Patient no.	HIV RNA (copy no./ml)[c]		$CD4^+$ T lymphocytes (cells/mm^3)	
	Pre[d]	Post[e]	Pre	Post		Pre	Post	Pre	Post
1	—[f]	—	250	146	1	—	—	574	493
2	1,271 ± 86	—	403	592	2	925 ± 35	—	436	577
3	892 ± 67	—	183	249	3	4,497 ± 350	—	242	66
4	4,740 ± 305	—	498	297	4	6,740 ± 177	1,100 ± 74	212	386
5	903 ± 29	—	272	200	5	4,985 ± 21	793 ± 29	360	539
6	9,940 ± 509	1,160 ± 57	376	768	6	4,606 ± 427	953 ± 46	310	350
7	4,725 ± 318	1,065 ± 52	530	685	7	4,470 ± 181	922 ± 52	389	403
8	4,635 ± 268	1,007 ± 15	125	165	8	4,700 ± 250	1,065 ± 92	250	354
9	5,269 ± 914	1,037 ± 57	626	421	9	4,700 ± 396	955 ± 78	297	147
10	6,253 ± 296	1,033 ± 31	315	240	10	1,020 ± 58	940 ± 35	377	320
11	5,013 ± 187	925 ± 18	270	118	11	987 ± 23	4,103 ± 81	418	356
12	1,083 ± 76	767 ± 29	411	405					
13	1,013 ± 25	817 ± 110	320	139					
14	807 ± 15	787 ± 23	270	110					
15	4,090 ± 127	9,400 ± 367	156	96					
Mean for group ± SEM	3,617 ± 756	1,800 ± 845	333 ± 36	313 ± 56		3,763 ± 642	1,353 ± 394[g]	351 ± 31	363 ± 46

[a] Fifteen HIV-positive patients receiving combination treatment with ZDV plus alpha interferon for 48 weeks.
[b] Eleven HIV-positive patients treated with ZDV alone for 48 weeks.
[c] Results are expressed as means ± SEM of three independent quantifications.
[d] Pre, before antiretroviral therapy.
[e] Post, after antiretroviral therapy.
[f] —, not detectable (<50 copies of HIV-1 RNA per ml).
[g] Posttherapy values significantly different from pretherapy values at $P < 0.05$.

$P < 0.01$) done on the same day. When the same serum samples were extracted on different days, the correlation of mean absorbance was somewhat less ($r = 0.80$, $P < 0.01$). When intra-assay variability of the nested PCR was tested, multiple replicates of the same PCR sample yielded absorbances, as determined by densitometric scanning, that varied <15% between lanes of the agarose gel. Furthermore, the quantitation of duplicate samples extracted and amplified on different days showed an interassay variability of 20 to 25%.

To make the measurements of HIV RNA more reliable, we always analyzed serum samples obtained at different times from one patient in the same PCR assay. The PCR assays always included the standard curve for known amounts of a stock of HIV virus in 200 μl of HIV-seronegative control serum (Fig. 1B).

Detection and quantification of HIV RNA in the serum samples of 26 p24 antigen-negative, asymptomatic, HIV-positive IDUs undergoing antiretroviral therapy. To quantify the number of copies of viral RNA present in patients' sera, decreasing volumes of serum (1,000, 200, 40, 10, and 2 μl) from each patient were extracted and reverse transcribed, and the nested PCR was performed as indicated above. After PCR, the intensity of the specific band (341 bp) was quantified with a densitometer, as mentioned above. The absorbance value that corresponds to the smallest volume of serum which gives a specific band was then extrapolated to a standard curve of HIV S61 virus run in the same PCR assay to calculate the number of copies of HIV RNA.

HIV p24 antigen assay. To improve the detection of the levels of HIV p24 antigen in serum, the circulating immune complexes were dissociated by a method based on low-pH treatment of the patients' samples before use of an enzyme-linked immunoassay (ELAVIA-Ag I; Diagnostics Pasteur) (6). Briefly, 4 volumes of serum are mixed with 1 volume of 0.5 N HCl. After incubation of the mixture for 90 min at room temperature, the treated serum is neutralized with 1 volume of 0.5 N NaOH, mixed, and immediately added to two wells of the reaction microplate. After this treatment of the patients' sera, the enzyme-linked immunoassay procedure was performed according to the manufacturer's instructions. At enrollment in the study, none of the 26 HIV-positive, asymptomatic IDUs showed detectable p24 antigen in serum.

Quantification of T-cell subsets in peripheral blood. T-lymphocyte subsets in peripheral blood were quantified by direct immunofluorescence using monoclonal antibodies of the T series and flow cytometry (FACScan; Becton-Dickinson) as previously described (5).

Statistics. The statistical differences in the quantities of HIV RNA within the combination therapy and the monotherapy groups and between the two groups, before and after treatment, were analyzed by a nonparametric test (Mann-Whitney, Wilcoxon), since the data did not show a normal distribution. The differences in the absolute numbers of $CD4^+$ T cells were analyzed by the Student t test. Correlations were studied by using Pearson's linear regression analysis.

RESULTS

We have used a nested-PCR method for the detection and quantification of HIV RNA in the serum samples of 26 p24 antigen-negative, asymptomatic, HIV-positive IDUs undergoing antiviral therapy. Before the beginning of the antiviral treatment, a specific band was detectable in 24 of the 26 serum samples (92%) (Table 1). After 48 weeks of therapy, a fall in the viral titer was observed for patients in the combination therapy group (mean titers of copies of HIV RNA ± standard errors of the means [SEM], 3,617 ± 756 pretherapy versus 1,800 ± 845 posttherapy; $P < 0.05$) and patients in the monotherapy group (3,763 ± 642 pretherapy versus 1,353 ± 394 posttherapy; $P < 0.05$) (Table 1). However, there were no significant differences, after 48 weeks, in the reduction of viral load between the two therapy groups. In the combination therapy group, the HIV RNA in patients' serum samples was decreased in 12 (80%), unchanged in 2 (13%), and increased in 1 (7%). In the monotherapy group, the HIV viremia was decreased in eight samples (73%), unchanged in two samples (18%), and increased in one sample (9%) (Table 1).

The 26 serum samples analyzed in this study were from p24 antigen-negative, asymptomatic, HIV-positive IDUs; consequently, most of the patients showed low levels of RNA viremia (fewer than 5,000 copies of HIV RNA per ml). In this study, we also analyzed, by the nested-PCR assay, serum samples from seven patients with p24 antigenemia. The results showed that five of the seven patients (mean p24 antigen level ± SEM, 378 ± 132 pg/ml) showed viral titers of >10,000 copies of HIV RNA per ml. The other two patients (mean p24 antigenemia ± SEM, 225 ± 106 pg/ml) showed lower levels of HIV viremia (9,720 and 8,900 copies of HIV RNA per ml).

We have also quantified the absolute numbers of $CD4^+$ lymphocytes in the peripheral blood of the 26 HIV-positive IDUs (Table 1). There were no significant differences in the

absolute numbers of $CD4^+$ T cells between the two treatment groups, either at the beginning of therapy (mean ± SEM, 333 ± 36/mm^3 for the combination therapy group versus 351 ± 31/mm^3 for the monotherapy group) or after 48 weeks of therapy (mean ± SEM, 313 ± 56/mm^3 for the combination therapy group versus 363 ± 46/mm^3 for the monotherapy group). Also, within each therapy group, the changes in the absolute numbers of $CD4^+$ cells before and after treatment were not significantly different.

We have investigated the relationship between the changes in the levels of HIV RNA in patients' sera and the changes in the absolute numbers of $CD4^+$ T cells after 48 weeks of antiviral treatment (Table 1). Of the 24 HIV-positive patients with viremia at the beginning of treatment ($n = 24$), 11 showed an increase in the number of $CD4^+$ T cells and a decrease in the RNA viremia. Of the 13 patients who showed a decrease in the number of $CD4^+$ cells, the RNA viremia decreased in 11 and increased in 2. There was not a significant association between the changes in the number of $CD4^+$ T cells and the changes in the level of HIV RNA in serum.

DISCUSSION

There is no standard method with which to assess viremia in HIV-positive patients at different stages of HIV infection. Fast and reproducible assays for the quantification of HIV RNA are becoming increasingly important for early diagnosis of HIV infection and for accurate assessment of the efficacy of antiviral therapy. Quantitative cultures allow the detection and quantification of infectious virus in the serum of about 50 to 100% of HIV-positive patients, mostly at advanced stages of HIV infection, with low numbers of $CD4^+$ T cells and with p24 antigenemia (2, 4). More recently, by using qualitative PCR assays, it has been demonstrated that the majority of HIV-infected patients have detectable HIV RNA in the plasma (10, 16). However, those PCR assays are not readily applicable to the quantification of HIV RNA in serum.

Using a nested PCR, we have detected and quantified HIV RNA in the sera of the majority (92%) of the asymptomatic, HIV-positive IDUs negative for p24 antigenemia and with a low viral load in this study. Changes in serum HIV RNA levels were detected in 13 of the 15 patients (87%) undergoing combination therapy and in 9 of the 11 patients (82%) in the monotherapy group.

Reproducible quantification of viral RNA was achieved by this nested PCR over a range of 10 to 1,000 HIV-1 RNA copies per 200 μl within a linear range, with as few as 50 viral particles per ml of a stock of HIV-1 in a small volume of serum being detected.

The level of sensitivity of this nested PCR is similar to that of other techniques previously reported (7, 14). For example, it has been shown that a PCR coupled to a nonisotopic enzyme-linked assay can detect a minimum of 100 copies of HIV-1 RNA per ml (7). Other authors have demonstrated the ability of the PCR method to detect 100 copies of HIV RNA per ml by using 30 cycles of amplification of an HIV-specific sequence and hybridization with an isotopically labeled probe (2). Another study using a quantitative competitive PCR method has shown a similar sensitivity (100 RNA copies per ml) (14). However, the PCR technique described above and other PCR techniques used by other investigators do not provide the level of stringent internal control (which is a central feature of quantitative competitive PCR) required to provide a rigorous basis for absolute quantitation (14). In the studies mentioned above, the detection of RNA in the sera of HIV-positive patients varied from 69 to 96%. Those studies used a heterogeneous group of patients with respect to the clinical stages of the HIV infection and the presence of p24 antigenemia. Levels of HIV-1 RNA higher than 1,000 eq/ml are readily detected by a nucleic acid hybridization assay based on signal amplification branched-DNA technology.

A potential problem with the high degree of sensitivity of the nested-PCR assay is that minute contamination of samples or reagents by target DNA will be amplified and cause a positive score (11). To overcome this problem, we undertook strict laboratory routines (11). Furthermore, HIV-infected samples were never handled in the laminar-airflow cabinet used for preparation of the samples for PCR. Also, plasmid DNA or PCR products have never been used in our PCR laboratory.

Finally, our data suggest that the determination of virion-associated HIV-1 RNA levels in serum by PCR represents a marker of viral replication with potential for widespread applicability in the assessment of the efficacy of antiretroviral therapy. There is a widely recognized need for new markers that would allow timely assessment of the in vivo antiviral activity of new therapeutic approaches and agents. Future studies will also help to determine the relationship between virion-associated HIV RNA levels and clinical outcome of the HIV infection.

ACKNOWLEDGMENTS

We thank Thomas C. Merigan for fruitful discussions and critical review of the manuscript and Dolores Garcia for her excellent technical assistance. HIV-1 S61 was a gift of C. Lopez-Galíndez from Instituto de Salud Carlos III.

This work was supported by grant C134/90 from the Autonomous Government of the State of Madrid, "Plan Regional de Investigación."

REFERENCES

1. **Albert, J., and E. M. Fenyo.** 1990. Simple, sensitive, and specific detection of human immunodeficiency virus type 1 in clinical specimens by polymerase chain reaction with nested primers. J. Clin. Microbiol. **28:**1560–1564.
2. **Becker-Andre, M., and K. Hahlbrock.** 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res. **17:**9437–9446.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Coombs, R. W., A. C. Collier, J. P. Allain, B. Kikora, M. Leuther, G. F. Gjerset, and L. Corey.** 1989. Plasma viremia in human immunodeficiency virus infection. N. Engl. J. Med. **321:**1626–1631.
5. **Fernández-Cruz, E., M. Desco, M. García Montes, N. Longo, B. González, and J. M. Zabay.** 1990. Immunological and serological markers predictive of progression to AIDS in a cohort of HIV-infected drug users. AIDS **4:** 987–994.
6. **Gonzalez, B., M. García Montes, J. M. Zabay, N. Longo, J. M. Sempere, and E. Fernández-Cruz.** 1991. Circulating immune complexes (CIC) in HIV-infected drug users (IVDU). Demonstration of p24 antigen as a component of CIC in patients with and without free-antigen throughout the course of HIV infection, abstr. W. A. 1141, p. 127. *In* Abstracts of the VIIth International Conference on AIDS, Florence, Italy, 1991.
7. **Holodniy, M., D. A. Katzenstein, D. M. Israelski, and T. C. Merigan.** 1991. Reduction in plasma human immunodeficiency virus ribonucleic acid after dideoxynucleoside therapy as determined by the polymerase chain reaction. J. Clin. Invest. **88:**1755–1759.
8. **Holodniy, M., D. A. Katzenstein, S. Sengupta, et al.** 1991. Detection and quantification of human immunodeficiency virus RNA in patient's serum by use of the polymerase chain reaction. J. Infect. Dis. **163:**862–866.
9. **Holodniy, M., M. A. Winters, and T. C. Merigan.** 1992. Detection and quantification of gene amplification products by a nonisotopic automated system. BioTechniques **12:**38–39.
10. **Jurriaans, S., J. T. Dekker, and A. de Ronde.** 1992. HIV-1 viral DNA load in peripheral blood mononuclear cells from seroconverters and long-term infected individuals. AIDS **6:**635–641.
11. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339:**237–238.
12. **Munoz, A., M. C. Wang, S. Bass, J. M. Taylor, L. A. Kingsley, J. S. Chmiel, and B. F. Polk.** 1989. Acquired immunodeficiency syndrome (AIDS)-free time after human immunodeficiency virus type 1 (HIV-1) seroconversion in

homosexual men. Multicenter AIDS Cohort Study Group. Am. J. Epidemiol. **130:**530–539.
13. **Pantaleo, G., C. Graziosi, and A. S. Fauci.** 1993. New concepts in the immunopathogenesis of human immunodeficiency virus infection. N. Engl. J. Med. **328:**327–335.
14. **Piatak, M., M. S. Saag, L. C. Yang, et al.** 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science **259:**1749–1754.
15. **Saag, M. S., M. J. Crain, W. D. Decker, et al.** 1991. High-level viremia in adults and children infected with human immunodeficiency virus: relation to disease stage and $CD4^+$ lymphocyte levels. J. Infect. Dis. **164:**72–80.
16. **Scadden, D. T., Z. Wang, and J. E. Groopman.** 1992. Quantitation of plasma human immunodeficiency virus type 1 RNA by competitive polymerase chain reaction. J. Infect. Dis. **165:**1119–1123.