Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 1-8

QUANTITATIVE ANALYSIS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 DNA IN ASYMPTOMATIC CARRIERS USING THE POLYMERASE CHAIN REACTION

Shinichi Oka[1], Kyoko Urayama, Yoshihiro Hirabayashi, Kiyokata Ohnishi, Hajime Goto, Keiji Mitamura, Satoshi Kimura, and Kaoru Shimada

The Department of Infectious Diseases, the Institute of Medical Science, the University of Tokyo
4-6-1, Shirokanedai, Minato-ku, Tokyo, 108 Japan

Received January 4, 1990

A method for detecting human immunodeficiency virus type 1 (HIV-1) provirus DNA in lymphocytes with improved sensitivity and reproducibility was developed using the polymerase chain reaction(PCR). Amplified HIV-1 DNA was hybridized with a ^{32}P-labeled probe and quantitated with a beta-scanner after electrophoresis. A linear relationship was obtained between the common logarithms of the counts detected and the number of HIV-1 DNA copies applied to the PCR. Detectability was from 3 copies/10^5 lymphocytes, and linearity was maintained from 10 to 10^3 copies. HIV-1 DNA was detected in all 9 asymptomatic carriers tested (18 to 2,857 copies/10^5 CD4+ T lymphocytes). The viral burden was inversely related to the CD4+ lymphocyte count, suggesting that quantitation of provirus levels may serve as a predictor of progress in early HIV infection.

The polymerase chain reaction (PCR) is a rapid and highly sensitive assay for detecting human immunodeficiency virus type-1 (HIV-1) (1,2), which has been applied to diagnostic investigations (3-7). However, application of the technique for evaluation of the effects of antiviral therapy or direct assessment of the extent of the disease still limited by the lack of a simple and reliable method to quantitate the amount of original DNA. Schnittman et al. tried to evaluate the amount of HIV-1 DNA in CD4+ T lymphocytes by autoradiography of amplified DNA hybridized in liquid solution (8). However,

[1]To whom correspondence should be addressed.

0006-291X/90 $1.50

the results gave only a rough estimate because the autoradiograms were judged visually. Moreover, the relationship between the amount of HIV-1 DNA applied and the intensity of the autoradiograms was not liner. Abbott et al. developed a method to quantitate PCR-amplified products using slot-blot hybridization followed by densitometry scaning (9). Although this method allowed the results to be expressed as a numerical value, the sensitivity (over 80 copies per reaction) appeared not to be high enough for clinical use because the fraction of infected cells in the peripheral blood is very small. Improved PCR methods for quantitative analysis of the amount of HIV-1 DNA in infected individuals are urgently needed, especially when the effects of antiviral treatment are being evaluated in asymptomatic carriers in whom no other reliable marker of the viral burden is available.

We found a linear relationship between the common logarithms of the original amount of HIV-1 DNA and those of the radioactivity of amplified HIV-1 DNA (hybridized with a ^{32}P-labeled probe) measured by a beta-scanner after electrophoresis. This new method was found to be highly sensitive and reproducible, and it enabled us to examine the viral burden in asymptomatic HIV-1 seropositive individuals.

MATERIALS AND METHODS

Standard dilution: NY-M10 cells, which are a chronically infected T cell clone containing one proviral copy per cell (10), were used as the HIV-1 DNA standard. (The NY-M10 cells were kindly provided by Dr. N. Kobayashi, Department of Virology and Parasitology, Yamaguchi University, Yamaguchi, Japan). They were serially diluted with PBS from 10^5 cells to 300 cells per tube at 4 C and pelleted. The cells were lysed with 500 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer containing 0.001% Triton X-100, 0.0001% SDS, and 600 µg/ml of proteinase K (Boehringer-Mannheim Corp., Mannheim, West Germany) by incubation for 1 hour at 56 C, followed by incubation for 15 min at 95 C (8). A 5-µl aliquot of the lysate from each dilution of NY-M10 cells was next spiked into 50 µl of lysate prepared similarly from 10^5 non-infected Molt-4 cells. Thus, the standard cell lyastes contained DNA from 10^3

to 3 NY-M10 cells in the presence of excess control Molt-4 cell DNA.

Amplification: The lysates were subjected to 30 cycles of amplification with the PCR reaction mixture, as described elsewhere (8). The 3 primer pairs used in these experiments included SK38/39 (GAG), SK68/69 (ENV), and QH26/27 (HLA-DQa; control), also as described previously (2). After amplification, 20-µl aliquots were taken from HLA-DQa tubes and subjected to agarose gel electrophoresis (3% NuSieve, 1% agarose) to confirm whether or not the same amount of DNA had been amplified. Aliquots (30 µl) taken from tubes amplified with HIV-1 primer pairs were subjected to liquid hybridization with a ^{32}P end-labeled probe, as previously described (8).

Quantitation of amplified DNA: After electrophoresis on 10% polyacrylamide gel, the gel was scanned using an AMBIS Beta Scanning System (Automated Microbiology Systems Inc., San Diego. Calif.). To confirm the results obtained by scanning with AMBIS, the gels were exposed to Kodak XAR film at -70 C for 12 hours with an intensifying screen.

Patients: The amount of HIV-1 provirus DNA was quantitated using the method described above in 9 asymptomatic HIV-1 carriers, comprising 6 hemophiliacs, 2 homosexual men, and a heterosexually infected woman. Non of the patients had received any anti-HIV drugs such as azidothymidine or interferon. Patient no. 1 had seroconverted for HIV-1 between February and August 1989. Patients no. 2, 3, 4, 5, 8, and 9 were hemophiliacs who had been infected with HIV-1 between 1982 and 1986. They had remained asymptomatic for over 4 years, except for patient no. 9 who developed localized herpes zoster in 1986. For patients no. 6 and 7, it was not certain when they were infected with HIV-1. The mean CD4+ T lymphocyte count of these patients was 445 $\pm$ 73.6/µl (mean $\pm$ SEM).

Clinical samples: Blood samples were taken directly in heparinized syringes and subjected to Ficoll-Hypaque separation. One million peripheral blood mononuclear cells (PBMC) were washed twice and pelleted in PBS. The cells were lysed in the same manner as for the NY-M10 cells, and the lysates were stored at 4 C until use. Fifty µl of the lysate (one tenth of the lysed PBMC) was used in the PCR. Amplified DNA was quantitated by comparison with the standard samples containing known amounts of HIV-1 DNA prepared from NY-M10 cells as described above. Between the 2 results, one obtained with SK38/39 and the other with SK68/69, the larger number of HIV-1 DNA copies in 1 x 10^5 PBMC was taken as the number of HIV-1 DNA copies in each patient.

HIV p-24 antigen: The HIV-1 p-24 antigen levels in sera obtained at the same time were measured by enzyme-linked immunosorbent assay (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacture's instructions.

RESULTS AND DISCUSSION

A good linear relationship was repeatedly obtained between the common logarithms of the number of counts (cpm) of the radioactivity associated with each band of amplified HIV-1 DNA fragments and those of the number of HIV-1-infected cells (NY-

M10) over the range from 10^3 to 10^1. A representative regression line and an autoradiogram from the same experiment with the SK38/39 primer (hybridized with SK19) are shown in Figs. 1 and 2. The observation was highly reproducible. The intra-assay coefficients of variation at HIV-1 DNA concentrations equivalent to 10^3 and 10^1 infected cells were 2.8% and 18.7%, respectively (n=5). The inter-assay coefficient of variation was 4.2% when HIV-1 DNA from 10^2 infected cells was measured 5 times on different days. Samples containing as few as 3 copies of HIV-1 DNA gave counts significantly above the background. Similar results were obtained with SK68/69 primer pairs and the respective probe. HLA DQ-alpha DNAs was derived from 1 x 10^5 Molt-4 cells and relatively small numbers (10^3 - 3) of NY-M10 cells. The DNA was amplified equally in

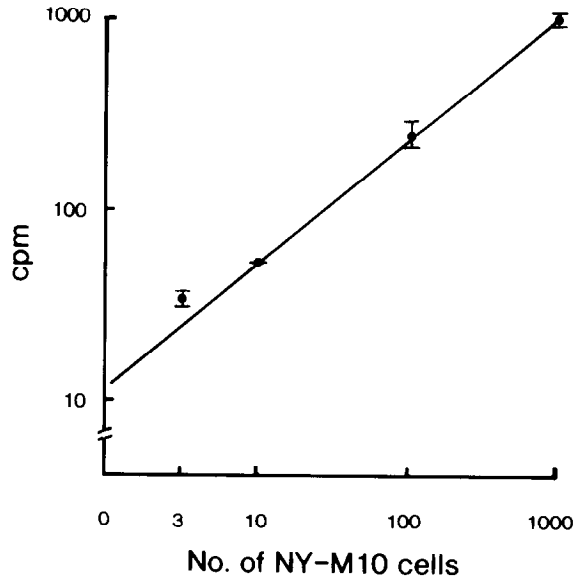
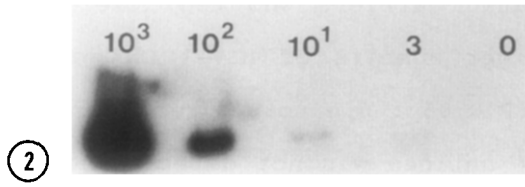


Figure 1: A regression line obtained from standard dilutions of NY-M10 cells, which contain one HIV-1 DNA copy per cell. After amplification and liquid hybridization, samples were subjected to PAGE. Gels were scanned by an AMBIS beta-scanner and the radioactivity of the corresponding bands was quantitated as cpm. The primer pair used was SK38/39. Each point for the standard represents the mean of duplicate samples. Bars and vertical lines indicate individual data.



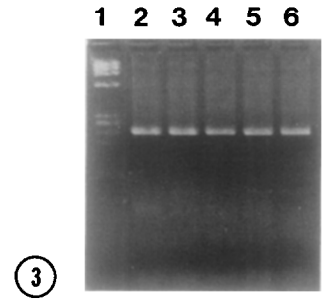


Figure 2: An autoradiogram (12-hour exposure) obtained from the same gel used to give the results shown in Fig. 1. Numbers at the top of each lane indicates the number of NY-M10 cells added to the lysate.

Figure 3: Amplification of HLA DQ-alpha. lane 1; phage X 174/Hae III digest. lane 2 - lane 6; each lane contained Molt-4 cell DNA derived from 10^5 cells and DNA obtained from 10^3 (lane 2), 10^2 (lane 3), 10 (lane 4), 3 (lane 5), and 0 (lane 6) NY-M10 cells, respectively. White bands in lanes 2 through 6 represent amplified DNA.

all samples, indicating that the amount of DNA applied and the magnitude of the amplification were the same (Fig. 3).

The results of the investigation of amplified HIV-1 DNA levels in clinical samples are summarized in Table I. The number of HIV-1 DNA copies per 10^5 PBMC was 200 or less. When the number of CD4+ T lymphocytes in each patient was taken into consideration, the number of HIV-1 DNA copies per 10^5

Table I. Viral burden in asymptomatic HIV-1-infected subjects who had not received any anti-HIV drugs

Patient no.	Risk factor	CD4+[a] (/μl)	HIV/10^5PBMC[b] SK38/39	SK68/69	HIV/10^5CD4+[c]	p24-Ag
1.	homosexual	631	93	ND	423	-
2.	hemophilia	336	14	93	266	-
3.	hemophilia	315	33	50	217	-
4.	hemophilia	535	42	<10	91	-
5.	hemophilia	393	<10	<10	<21	-
6.	heterosexual	715	38	-	106	-
7.	homosexual	744	<10	-	<18	-
8.	hemophilia	201	117	50	509	-
9.	hemophilia	138	200	18	2857	-

a) number of CD4+ T lymphocytes in peripheral blood.
b) number of HIV-1 DNA copies in 10^5 peripheral blood mononuclear cells.
c) number of HIV-1 DNA copies in 10^5 CD4+ T lymphocytes.
ND; not done, -; negative.

CD4+ T lymphocytes was calculated to range from less than 18 to 2,857 copies (median : 217 copies/10^5 CD4+ T lymphocytes). It was found that asymptomatic carriers who had lower CD4+ counts had significantly larger numbers of HIV-1 DNA copies per 10^5 CD4+ T lymphocytes ($P<0.05$: Spearman's rank correlation coefficient). The p-24 antigen was not detected in any of the subjects.

HIV-1 DNA in clinical samples has been also detected by Southern blot hybridization (11). However, because of the extremely small quantity of proviruses present in clinical samples, the method is not sensitive enough to assess the progress of the disease. The appearance of the p-24 antigen has been associated with progression of the disease, and the antigen level has been shown to parallel viral activity (12, 13). Therefore, it can be used as a virological marker in the evaluation of the effects of anti-HIV drugs (14,15). However, most asymptomatic carriers are negative for p-24 as well for the isolation of the virus. Thus, until now there have been no virological markers when clinical trials were conducted in an early-stage HIV-1 infection, which makes the evaluation of such trials very difficult (16).

In the present study, HIV-1 DNA levels could be quantitatied in most of the asymptomatic carriers tested. The method is simple, rapid, sensitive enough for clinical use and reproducible. This test provides an objevctive measure of the extent of HIV infection, and quantitation of HIV provirus levels should be useful for monitoring the clinical effects of antiviral agents, especially in the early stages of the disease.

All patients in this study were asymptomatic and had not been treated with any drugs. Under such conditions, the

viral burden was found to show an inverse relationship to the CD4+ count. This observation is compatible with the notion that a higher viral burden contributes to a decline in the CD4+ T lymphocyte count. Therefore, quantitation of provirus levels may be a useful predictor of the progress of the disease in the early stage. Further studies can be expected to reveal whether and/or when anti-HIV therapy should be used in asymptomatic carriers.

ACKNOWLEDGMENTS

We wish to thank Drs. S. M. Schnittman and M. C. Psallidopoulos for providing primer pairs and probes and for their helpful suggestions. Dr. N. Kobayashi is also acknowledged for providing NY-M10 cells. This work was supported in part by a Grant-in-Aid for AIDS Research from the Ministry of Health and Welfare and from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1) Kwok, S., Mack, D. H., Mullis, K. B., Poiesz, B., Ehrlich, G., Blair, D., Friedman-Kien, A. and Sninsky, J. J. (1987) J. Virol. 61, 1690-1694.
2) Ou, C. Y., Kwok, S., Mitchell, S. W., Mack, D. H., Sninsky, J. J., Krebs, J. W., Feorino, P., Warfield, D. and Schochetman, G. (1988) Science 239, 295-297.
3) Farzadegan, H., Polis, M. A., Wolinsky, S. M., Rinaldo, C. R., Sninsky, J. J., Kwok, S., Griffith, R. L., Kaslow, R. A., Phair, J. P. Polk, B. F. and Saah, A. J. (1988) Ann. Intern. Med. 108, 785-790.
4) Loche, M. and Mach, B. (1989) Lancet ii, 418-421.
5) Imagawa, D. T., Lee, M. H., Wolinsky, S. M., Sano, K., Morales, F., Kwok, S., Sninsky, J. J., Nishanian, P. G., Giorgi, J., Fahey, J. L., Dudley, J., Visscher, B. R. and Detels, R. (1989) N. Eng. J. Med. 320, 1458-1489.
6) Rogers, M. F., Ou, C. Y., Rayfield, M., Thomas, P. A., Schoenbaum, E. E., Abrams, E., Krasinski, K., Selwyn, P. A., Moore, J., Kaul, A., Grimm, K. T., Bamji, M., Schochetman, G. and The New York City Colllaborative Study of Maternal HIV Transmission and Montefiore Medical Center HIV Perinatal Transmission Study Group. (1989) N. Eng. J. Med. 320, 1649-1654.
7) Horsburgh, C. R., Ou, C. Y., Jason, J., Holmberg, S. D., Longini, I. M., Lifson, A. R., Schochetman, G., Ward, J. W., Rutherford, G. W., Evatt, B. L., SeageIII, G. R. and Jaffe, H. W. (1989) Lancet ii, 637-640.
8) Schnittman, S. M., Psallidopoulos, M. C., Lane, H. C., Thompson, L., Baseler, M., Massari, F., Fox, C. H., Salzman, N. P. and Fauci, A. S. (1989) Science 245, 305-308.
9) Abbott, M. A., Poiesz, B. J., Byrne, B. C., Kwok, S., Sninsky, J. J. and Ehrlich, G. D. (1988) J. Infect. Dis. 158, 1158-1169.

10) Hamamoto, Y., Takamatsu, K., Kobayashi, S., Yamaguchi, K., Yamamoto, N. and Kobayashi, N. (1898) Virus Genes 3, 141-152.
11) Shaw, G. M., Hahn, B. H., Arya, S. K., Groopman, J. E., Gallo, R. C. and Wong-Staal, F. (1984) Science 226, 1165-1171.
12) Goudsmit, J., Wolf, F. G., Paul, D. A., Epstein, L. G., Lange, J. M. A., Krone, W. J. A., Speelman, H., Wolters, E. C., Noordaa, J. V. D., Oleske, J. M., Helm, H. J. V. D. and Coutinho, R. A. (1986) Lancet ii, 177-180.
13) Goudsmit, J., Lange, M. A., Paul, D. A. and Dawson, G. J. (1987) J. Infect. Dis. 155, 558-560.
14) Spector, S. A., Kennedy, C., McCutchan, J. A., Bozzette, S. A., Strabe, R. G., Connor, J. D. and Richman, D. D. (1989) J. Infect. Dis. 159, 822-828.
15) Burger, H., Paul, D., Siegal, F. P., Wendel, I., Neff, S., Eilbott, D., Gehan, K., Grimson, G. and Weiser, B. (1988) J. Clin. Microbiol. 26, 1890-1892.
16) Oka, S., Hirabayashi, Y., Mouri, H., Sakurada, S., Goto, H., Ohnishi, K., Kimura, S., Mitamura, K. and Shimada, K. (1989) J. AIDS 2, 125-128.