Quantification of Human Immunodeficiency Virus Type 1-Infected Mononuclear Cells in Peripheral Blood of Seropositive Subjects by Newly Developed Flow Cytometry Analysis of the Product of an In Situ PCR Assay

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The presence of human immunodeficiency virus type 1 (HIV-1) proviral DNA in peripheral blood mononuclear cells (PBMC) of three groups (group 1, more than 500 CD4+ T cells per μl; group 2, between 200 and 499 CD4+ T cells per μl; group 3, fewer than 200 CD4+ T cells per μl) of HIV-1-infected patients, in different stages of the disease, was determined by using a newly developed flow cytometry analysis of the product of in situ PCR assay and compared with other markers of viral replication (HIV-1 p24 antigenemia and viral isolation). Results showed varied percentages of HIV-1-infected PBMC, ranging from 0.6 to 20%. Patients with more than 500 CD4+ T cells per μl showed the lowest percentage of HIV-1-infected PBMC (2.1 ± 1.7), compared with patients with CD4+ T-cell counts of between 200 and 499 per μl (6.5% ± 4.1%; $P < 0.001$) and patients with fewer than 200 CD4+ T cells per μl (4.9% ± 4.7%; $P < 0.05$). The difference in the percentage of HIV-1-infected PBMC between group 2 and group 3 patients may in part reflect the loss of CD4+ T lymphocytes in more advanced stages of the disease. However, the results clearly indicate a striking coincidence between the fall of the CD4+ T-cell count below 400/μl and the sharp increase in PBMC virus loading and p24 antigenemia. Since the procedure is relatively easy to perform, it could be used to monitor the evolution of HIV-1 infection and may prove a useful adjunct in tailoring therapeutic strategies.

A dramatic decrease in the number of circulating CD4+ helper-inducer T lymphocytes, followed by a profound derangement of the immune response, is the hallmark of disease progression in human immunodeficiency virus type 1 (HIV-1)-infected patients (17, 21, 22, 25).

The quantification of peripheral blood mononuclear cells (PBMC) productively or latently infected by HIV-1 is essential to understand various pathogenic aspects of the disease, better define the stage of the disease, and tailor therapeutic strategies.

Different technical procedures have been used to assess the frequency of HIV-1-infected cells in the bloodstream (5, 28), lymphonodi (15, 20), and other regions of the body (17). Although some studies have furnished interesting new data, conflicting results have been reported. While initial studies demonstrated that only 1 or fewer in 100 CD4+ T cells harbored HIV-1 provirus (5, 27–29), subsequent research suggested that the percentage of infected PBMC may be much higher than previously described (1–4, 9, 15, 23, 26).

The different percentages reported by different authors may be due, in part, to technical differences in the various studies. A current limitation of PCR on extracted DNA is the difficulty in correlating the presence of the viral genome with a single cell and thus determining the exact percentage of HIV-1-carrying cells (13). On the other hand, the in situ hybridization procedures suffer from low sensitivity and although in situ PCR can detect a single infected cell, it requires microscopical screening of a large number of cells to determine the exact percentage of HIV-1-harboring cells (1, 18). The combination of in situ PCR and flow cytometry analysis, instead, can overcome most these drawbacks (23, 31).

Knowing the percentage of HIV-1-infected PBMC during the different stages of the disease may provide important information. We therefore investigated the presence of HIV-1 proviral DNA in the PBMC of three groups of HIV-1-seropositive patients, using a newly developed flow cytometry analysis of the product of in situ PCR assay in comparison with other markers (HIV-1 p24 antigenemia and virus isolation) of infection.

MATERIALS AND METHODS

Patients and controls. Peripheral blood samples were obtained from 53 HIV-1-seropositive subjects classified on the basis of the 1993 Centers for Disease Control revised classification system for HIV infection (7) and from 15 healthy blood donors, after informed consent according to the Helsinki Declaration of 1975.

Group 1 comprised 14 patients who were asymptomatic or showed only persistent generalized lymphadenopathy with a mean CD4+ T-lymphocyte number of (696 ± 200) × 10⁶/liter. Group 2 included 15 symptomatic subjects, with no AIDS indicator conditions, with a mean CD4+ T-lymphocyte number of (347 ± 74) × 10⁶/liter. Group 3 comprised 24 patients in an advanced stage of the disease, with a mean CD4+ T-lymphocyte number of (76 ± 68) × 10⁶/liter.

Three of the 14 asymptomatic patients, 7 of the 15 with symptomatic disease, and 10 of the 24 with AIDS were

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undergoing 3'-azido-3'-deoxythymidine (zidovudine) treatment when the peripheral blood samples were obtained (Table 1).

Cell separation. Venous blood was collected in EDTA (K_3, 7.5%) Vacutainers (Becton Dickinson) and processed within 4 h. Low-density PBMC were isolated by collecting the floating fractions obtained from leukocyte pellets after centrifugation over Ficoll-Hypaque density gradients (Sigma, St. Louis, Mo.) (24). These cells were immediately cultured for virus isolation and for PCR assays as described below.

HIV-1 p24 antigenemia and viral isolation. Plasma samples from HIV-1-infected patients were immediately frozen at −70°C. HIV-1 p24 antigenemia, after immune complex dissociation by acid treatment, was evaluated by a commercial enzyme-linked immunosorbent assay kit for the specific quantitative determination of HIV-1 *gag* p24 antigen (Du Pont de Nemours) used according to the manufacturer's instructions. Positive results were all confirmed by neutralization tests (HIV-1 p24 confirmatory test; Du Pont de Nemours). The concentration of HIV-1 *gag* p24 antigen for each positive sample was gauged by interpolation from the standard curve by quadratic regression methods. Plasma samples with p24 levels below 5 pg/ml were considered negative.

For isolation of infectious HIV-1, 10^6 low-density PBMC from patients' blood were kept in growth medium (RPMI 1640 medium; GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated (56°C for 45 min) fetal calf serum (GIBCO), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; 5 mM), and gentamicin (50 μg/ml) and stimulated with 1 μg of phytohemagglutinin (Wellcome HA 1617) per ml, at 37°C. After 2 days, cells cocultivated with 10^6 phytohemagglutinin-stimulated PBMC obtained from healthy donors were maintained in RPMI 1640 supplemented with 10% TCGF (Medical System).

Coculture supernatants were tested twice a week for the presence of HIV-1 *gag* p24 antigen.

In situ PCR. The reaction was carried out by a newly developed method of flow cytometry analysis of the product of in situ PCR assay. The method was derived, with slight modifications, from methods already described in reports from our (13, 16) and other (23, 31) laboratories and is based on a PCR carried out in the presence of digoxigenin-labeled dUTP to obtain a digoxigenin-labeled amplicon able to react with an antidigoxigenin fluorescein-conjugated antibody detectable by a flow cytometric procedure.

Briefly, the reaction was carried out as follows. Cells (5 × 10^5) fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 2 h at room temperature and washed three times with 500 μl of PBS were treated with 200 μl of pronase (10 μg/ml) for 5 min at 37°C in a water bath. Pronase inactivation was performed by placing the cells at 95°C for 3 min. The cell pellet, obtained by centrifugation at 1,500 × g for 5 min at room temperature, was resuspended in 50 μl of the following PCR mixture: 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 220 μM (each) dATP, dCTP, and dGTP, 215 μM dTTP, 7 μM digoxigenin-labeled dUTP, 200 pM each primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Oligonucleotide primers SK38 and SK39, which can amplify a 115-bp region of the HIV-1 *gag* gene, were used (19, 30). The amplification was carried out by a hot-start technique (17) to prevent mispriming and primer dimerization. The sample was subjected to 35 cycles of denaturation, reannealing, and extension, which were performed at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1.5 min, respectively. At the end of the reaction, the cells were washed twice with PBS (200 μl) and treated with 1% blocking reagent (Boehringer

GmbH, Mannheim, Germany) in PBS (200 μl) for 10 min at 37°C.

Samples were then centrifuged at 1,500 × g for 5 min and resuspended in 200 μl of 1% blocking reagent. Antidigoxigenin fluorescein-conjugated antibody (Boehringer GmbH) was added (final dilution of 1:1,000), and samples were incubated for 30 min at room temperature. Finally, after several washings with 200 μl of PBS, the samples were analyzed by flow cytometry.

To assess the DNA amplification capability and positive signal detection in the system employed, samples were analyzed in parallel with oligonucleotide primers GH26 and GH27 (Perkin-Elmer Cetus), which flank a conserved region of the HLA-DQ (α) locus, as previously described (12). A couple of lambda phage-specific oligonucleotide primers (5'-GATGA GTTCGTGTCCGTACAACTGG-3', mapping at positions 7131 to 7155, and 5'-GGTTATCGAAATCAGCCACAGCG CC-3', mapping at positions 7606 to 7630) which have no homologies with HIV-1 or cellular DNA were used as negative controls.

Construction of a standard curve and positive control for PCR assays. The 8E5/LAV cell line (11), which contains a single copy of the HIV-1 provirus genome per cell, was used as a positive control to verify both the specificity and sensitivity of the test.

Cells were passed twice a week in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO).

Cells in the mid-log phase of growth were washed in PBS and counted in a hemocytometer. To assess the lowest limit of positive signal detection by the procedure employed and to construct a standard curve for PCR assays, 8E5/LAV cells were mixed with uninfected lymphoblastoid A3.01 cells (10) in various proportions up to a total of 5 × 10^5 cells and stored at 4°C until tested by PCR as previously described.

Flow cytometry. Cells were analyzed with a FACScan (Becton Dickinson, Baltimore, Md.) flow cytometer. Laser excitation was 15 mW at 488 nm.

Instrument sensitivity was standardized with calibration beads (Becton Dickinson). The percentage of fluorescent positive cells was determined by integration over a range of 0.1% positive counts on identically treated negative controls (100% uninfected PBMC) or on cell samples for which the in situ PCR had been performed with nonspecific primers. At least 10,000 cells were analyzed for each sample.

Statistical analysis. The results are expressed as means ± standard deviations of the data obtained in triplicate experiments. A two-tailed Student's *t* test for unpaired data was used for statistical comparison.

RESULTS

HIV-1 p24 antigen detection and viral isolation. Among the 14 patients of the first group, only 2 (14%) showed detectable levels of p24 (from 12.2 to 56.9 pg/ml) in plasma samples, while viral isolation was positive in five cases (36%). Five of 15 patients of the second group (33%) and 14 of 24 of the third group (58%) showed positive levels of p24 (from 10 to 53 and from 16 to 560 pg/ml, respectively), while a positive viral isolation result was obtained from 12 of 15 patients in the second group and all patients in the third group (Table 1).

Standard curve and positive control for PCR assay. Uninfected lymphoblastoid A3.01 cells mixed with serially increasing percentages (0, 6.2, 12.5, 25, 50, and 100) of 8E5/LAV cells were subjected to amplification and flow cytometry analysis to verify the specificity of the method. A standard curve was plotted as shown in Fig. 1; data are representative of two

TABLE 1. Hematological and virological data from HIV-1-infected patients by CD4+ T-lymphocyte count

CD4+ T-cell count per liter and patient no.	No. of leukocytes (10⁶/liter)	Lymphocyte content (%)	Monocyte content (%)	No. of T lymphocytes (10⁶/liter)		p24 Ag[a] (pg/ml)	Detection by:		CD4+ cells/PBMC (%)
				CD4+	CD8+		Virus isolation	ISPCR[b] (%)	
>500 × 10⁶									
1	5,010	36.9	0.8	1,080	696		−	7.3	59.3
2[c]	7,100	44.8	6.0	1,024	1,006		−	1.0	40.2
3	8,100	55.0	15.7	945	2,920		+	2.8	38.7
4	6,400	40.2	4.9	858	1,014		−	0.8	40.6
5	5,800	47.6	5.1	756	1,132		+	3.0	34.4
6	4,700	46.3	7.2	638	836		−	0.6	38.8
7	4,800	41.2	8.8	620	1,080		−	0.8	43.4
8	5,100	38.6	6.0	580	980	56.9	+	0.8	39.0
9[c]	4,400	40.0	6.0	576	918		−	2.9	41.5
10[c]	6,000	28.9	7.3	561	714		−	0.8	46.0
11	4,800	36.0	9.0	544	833	12.2	+	2.3	45.2
12	4,800	35.5	7.2	527	816		−	2.1	42.6
13	3,800	35.7	7.0	520	1,200		+	1.7	48.4
14	3,500	45.9	5.9	512	576		−	2.2	39.6
Mean ± SD		40.9 ± 6.6	6.9 ± 3.2	696 ± 200	1,051 ± 566			2.1 ± 1.7	42.7 ± 6
(499–200) × 10⁶									
1[c]	7,700	24.8	5.8	480	912	10.0	+	2.4	39.3
2	9,800	36.4	6.4	432	2,736	29.2	+	2.2	25.3
3[c]	8,200	33.5	8.1	405	1,944		+	10.9	31.3
4	6,900	31.7	8.6	396	1,188		−	1.2	35.6
5[c]	6,400	42.0	8.0	378	1,755		+	11.3	27.8
6	4,900	45.0	6.0	374	1,232	53.0	+	11.0	26.7
7[c]	3,800	28.0	11.0	374	506		+	9.7	53.4
8	3,300	35.2	7.5	348	660		+	9.2	42.3
9[c]	4,500	39.8	10.7	342	1,116		+	3.0	36.2
10[c]	5,100	43.9	7.4	336	1,792		+	11.0	27.3
11	4,800	20.8	0.0	326	437		−	1.2	32.7
12[c]	4,500	46.7	7.7	315	1,140		−	9.1	27.0
13	6,100	52.7	6.5	256	1,760		+	7.7	18.1
14	3,200	29.8	10.1	240	530	19.2	+	1.3	44.1
15	8,700	23.0	3.0	200	1,520	15.1	+	6.0	20.4
Mean ± SD		35.6 ± 9.4	7.1 ± 2.8	347 ± 74	1,282 ± 643			6.5 ± 4.1	32.5 ± 9.5
<200 × 10⁶									
1[c]	4,570	39.0	12.0	190	1,390		+	5.6	31.7
2	5,200	51.0	7.0	189	2,079		+	1.2	18.3
3	3,900	53.1	3.0	189	1,197	74.3	+	7.4	14.0
4	5,000	37.0	6.0	180	1,296	423.0	+	8.0	22.3
5	3,200	40.0	6.5	143	793	33.7	+	20.0	23.6
6	8,400	27.7	4.7	138	1,863	204.0	+	4.6	19.6
7[c]	6,500	52.2	5.7	136	2,890		+	2.8	13.5
8	3,100	42.3	5.8	130	936	146.0	+	3.0	20.8
9	3,700	44.0	6.0	128	1,088	92.0	+	0.8	18.9
10	4,300	11.4	4.6	84	420	151.9	+	2.1	41.0
11	2,800	40.0	7.0	44	913	129.0	+	8.4	18.2
12[c]	2,700	41.1	10.9	44	484	140.0	+	2.3	24.1
13[c]	5,100	22.4	9.1	33	726		+	15.0	30.9
14[c]	3,700	28.0	8.0	33	638	560.0	+	6.3	24.7
15[c]	3,400	30.0	7.9	30	650		+	6.3	23.2
16	2,400	24.8	5.8	30	360	179.0	+	7.6	23.0
17	3,900	19.6	7.5	24	568	53.0	+	1.3	29.9
18	7,800	5.4	1.2	20	310		+	4.4	22.1
19[c]	5,700	32.7	5.2	19	1,501		+	2.3	14.6
20	3,400	27.0	3.0	18	648		+	1.0	11.8
21	2,100	25.7	8.0	15	325		+	1.0	25.9
22[c]	1,200	40.0	17.0	10	185	16.0	+	3.4	31.3
23[c]	3,200	14.8	4.7	6	444	149.0	+	0.8	25.1
24[c]	2,100	20.1	10.4	4	228		+	1.7	34.7
Mean ± SD		32.1 ± 12.8	7.0 ± 3.3	76 ± 68	914 ± 661			4.9 ± 4.7	23.5 ± 7.1

[a] Ag, antigen.
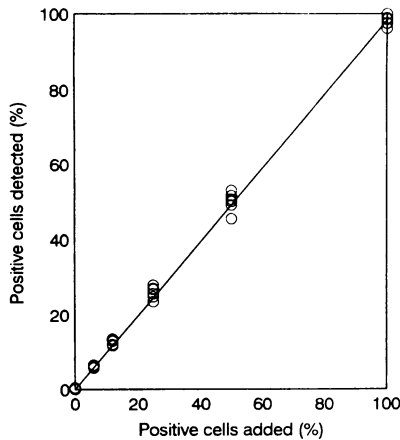[b] ISPCR, in situ PCR.
[c] Under zidovudine treatment.

FIG. 1. Diagram of results obtained in the experiments with uninfected lymphoblastoid A3.01 cells mixed with serially increasing percentages (0, 6.2, 12.5, 25, 50, and 100) of 8E5/LAV cells and subjected to amplification and flow cytometry analysis. Data are representative of two independent experiments run in triplicate.
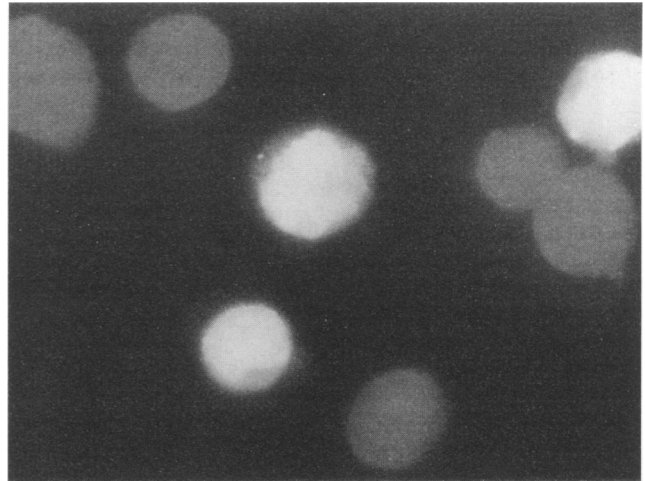
FIG. 3. Example of HIV-positive and -negative cells observed under a UV lamp-excited microscope. A delimited nuclear fluorescence can be observed.

independent experiments run in triplicate. The coefficients of variation at each experimental point never exceeded 5%. By a least-fit linear regression analysis a linear relation (correlation coefficient, $r = 0.998$) was observed between the percentage of single HIV-1 provirus-bearing cells added to each cell population and the percentage of positive cells detected.

Figure 2A to C show some representative histograms for

FIG. 2. The top histograms (A, B, and C) show some representative results obtained in the experiments with 8E5/LAV cells mixed with various percentages of uninfected A3.01 cells to assess the lower limit of sensitivity of the method employed: 0.05% 8E5/LAV cells plus 99.95% A3.01 cells (A); 40.00% 8E5/LAV cells plus 60.00% A3.01 cells (B); and 90.00% 8E5/LAV cells plus 10.00% A3.01 cells (C). The bottom histograms (D and E) show the percentages of HIV-1-infected PBMC observed in two widely different HIV-1-seropositive patients: 0.80% positive PBMC in a patient with an absolute CD4+ T-lymphocyte count of more than 500/µl (D) and 20.00% positive PBMC in a patient with an absolute CD4+ T-lymphocyte count of fewer than 200/µl (E).

mixed cell populations containing from 0.05 to 100% HIV-1 DNA-positive cells to ascertain the sensitivity of the test in our experimental conditions. A negative PCR signal was consistently obtained when 8E5/LAV cells were present at a frequency of <0.1%, which means that the lower limit for positive signal detection by the procedure was about 10 proviral genomes in a background of 10,000 cells.

No positive signal over the background was ever observed when DNA polymerase was omitted or lambda phage-specific primers were used. Nonspecific fluorescein-conjugated anti-digoxigenin antibody staining was not detected. Figure 3 shows an example of positive and negative cells with stained nuclei.

Percentage of HIV-1 provirus genome-containing PBMC detected by in situ PCR. As expected, all HIV-1-seropositive patients showed detectable levels of HIV-1 provirus DNA in PBMC, with percentages of positive cells ranging from 0.6 to 20%. Two representative histograms from patients with divergent values are illustrated in Fig. 2D and E.

The percentages of infected PBMC differed in the three categories of HIV-1-seropositive patients studied. The first group of subjects showed a mean value of infected PBMC of 2.1% ± 1.7% (with a range of positivity from 0.6 to 7.3%), while the other two categories presented mean values of 6.5% ± 4.1% and 4.9% ± 4.7% (with ranges of positivity from 1.2 to 11.3% and from 0.8 to 20.0%), respectively (Table 1).

These differences were significant between the first and second groups of patients ($P < 0.001$) and between the first and the third ($P < 0.05$). The differences remained significant when the percentages of infected PBMC were normalized for the CD4+ cells (T4 lymphocytes plus monocytes) estimated to be present in each sample analyzed by the flow cytometer (Fig. 4).

Figure 4 shows the normalized percentage of HIV-1 provirus-containing CD4+ cells and p24 antigenemia (picograms per milliliter) of each subject plotted against the absolute number of CD4+ T lymphocytes per microliter.

As can be seen, the percentage of virus-infected CD4+ cells and the absolute values of p24 antigenemia were consistently higher for patients with a CD4+ T-cell count below 400/µl than for patients having higher CD4+ T-cell counts.

No significant differences in the percentages of HIV-1-infected CD4+ cells were observed between the patients
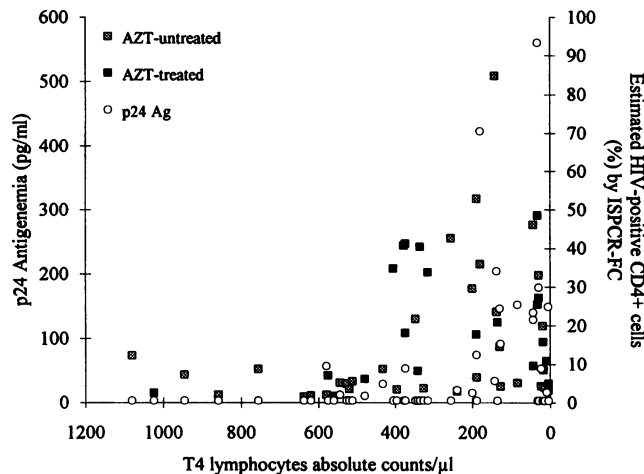
FIG. 4. Behavior of in situ PCR and flow cytometry (ISPCR-FC) normalized for CD4 counts on PBMC of zidovudine-treated and untreated patients and p24 antigenemia values plotted versus T4 absolute counts per microliter.

undergoing zidovudine therapy and those untreated at the time of blood sampling.

DISCUSSION

For different technical approaches, contrasting data have been reported on the number of infected PBMC and CD4+ T lymphocytes during the course of HIV-1 infection (2, 4, 5, 14, 28). The uncertainty of results and technical difficulties have so far prevented the routine use of virus load estimates for disease staging and tailoring of adequate therapeutic strategies.

By slight modifications of methods described in reports from our (13, 16) and other (23, 31) laboratories, we have worked out a procedure which allows the flow cytometric analysis of individual cells containing HIV-1 proviral DNA sequences amplified by in situ PCR. The procedure is based on the synthesis of a digoxigenin-tagged amplicon, by incorporation of digoxigenin-labeled dUTP, and its subsequent exposure to a fluorescein-conjugated antidigoxigenin antibody. This procedure is able to detect as few as 10 HIV-1 proviral genomes in a background of 10,000 cells.

By this method, we examined the percentage of HIV-1-infected PBMC in three groups (group 1, more than 500 CD4+ T cells per μl; group 2, between 200 and 499 CD4+ T cells per μl, group 3, fewer than 200 CD4+ T cells per μl) of HIV-1-infected patients in different stages of the disease and compared results with those of p24 antigenemia and virus isolation assays.

p24 antigenemia clearly correlated with the absolute number of CD4+ T cells per microliter and the stage of the disease, although it was only detected in a low percentage of patients. Virus isolation was constantly positive in almost all patients with CD4+ T-cell counts below 500/μl (Table 1). In agreement with those of other authors (8, 9, 14), our data showed that as the number of CD4+ T cells declines, the relative amount of HIV-1 p24 is higher and a greater number of subjects show HIV-1 p24 antigen detectable in plasma samples.

Flow cytometry analysis of the product of the in situ PCR assay performed on PBMC showed detectable levels of proviral DNA in all HIV-1-positive subjects studied. The percentage of positive cells significantly correlated with disease status, p24

antigenemia, and virus isolation when oligosymptomatic (group 1) and symptomatic (group 2 and 3) patients were taken into consideration (Table 1).

In agreement with the data reported by Hsia and Spector (13), the PBMC from patients with CD4+ T-cell counts between 200 and 499/μl frequently contained a higher percentage (and a higher absolute number) of HIV-1-infected cells than those with higher or lower CD4+ T-cell numbers.

No significant differences were observed between patients undergoing zidovudine treatment and those untreated in the three groups studied. This lack of correlation may be due to the limited number of subjects analyzed as well as to the length of the treatment period combined with different immunological parameters at the time of blood sampling. Thus our data suggest the need for further more specific longitudinal studies on HIV-1-positive patients monitored from the beginning of therapy onwards.

Although the lower percentage of HIV-1-infected PBMC in patients with fewer than 200 CD4+ T cells per μl compared with patients with CD4+ T-cell counts between 200 and 499/μl is not statistically significant and may be explained by the consistent loss of CD4+ T lymphocytes in the former group of subjects, our data demonstrate that the value around 500 CD4+ T cells per μl is a critical threshold below which a sharp increase in PBMC virus load is observed, while a number of CD4+ T cells per microliter below 200 is associated with evident virus replication, as demonstrated by the higher levels of p24 antigenemia (Fig. 4).

In most patients, however, the percentage of HIV-1-infected PBMC was relatively low, thus indicating that, besides HIV-1 infection of individual cells, additional mechanisms may contribute to the depletion of CD4+ T cells observed in vivo.

In conclusion, we have developed a relatively simple procedure which allows flow cytometry analysis of the product of an in situ PCR assay. With this technique, we analyzed the percentage of HIV-1-infected PBMC in 53 seropositive subjects, showing a fairly good correlation with CD4+ T-cell counts, the different stages of the disease, and other markers of HIV-1 infection. Moreover, the possibility of easily detecting the levels of transcriptionally active and quiescent viruses makes this a valuable method for monitoring the evolution of HIV-1 infection and providing a useful adjunct in tailoring of therapeutic strategies.

REFERENCES

1. Bagasra, O., S. P. Hauptman, H. W. Lischner, M. Sachs, and R. J. Pomerantz. 1992. Detection of human immunodeficiency virus type 1 provirus in mononuclear cells by in situ polymerase chain reaction. N. Engl. J. Med. 326:1385–1391.
2. Bagasra, O., and R. J. Pomerantz. 1993. Human immunodeficiency virus type I provirus is demonstrated in peripheral blood monocytes in vivo: a study utilizing an in situ polymerase chain reaction. AIDS Res. Hum. Retroviruses 9:69–76.
3. Bagasra, O., T. Seshamma, J. W. Oakes, and R. J. Pomerantz. 1993. High percentage of CD4-positive lymphocytes harbor the HIV-1 provirus in the blood of certain infected individuals. AIDS 7:1419–1425.
4. Bieniasz, P. D., K. Ariyoshi, M. A. Bourelly, S. Bloor, R. B. Foxall, E. C. Harwood, and J. N. Weber. 1993. Variable relationship between proviral DNA load and infectious virus titre in the peripheral blood mononuclear cells of HIV-1-infected individuals. AIDS 7:803–806.
5. Brinchmann, J. E., J. Albert, and F. Vartdal. 1991. Few infected

CD4+ T cells but a high proportion of replication-competent provirus copies in asymptomatic human immunodeficiency virus type 1 infection. J. Virol. **65**:2019–2023.

6. **Centers for Disease Control.** 1992. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. Morbid. Mortal. Weekly Rep. **41**:1–19.

7. **Chou, Q., M. Russell, D. E. Birch, J. Raymond, and W. Bloch.** 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res. **20**:1717–1723.

8. **Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase.** 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature (London) **362**:359–362.

9. **Folks, T., S. Benn, A. Rabson, T. Theodore, M. D. Hoggan, M. Martin, M. Lightfoote, and K. Sell.** 1985. Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. Proc. Natl. Acad. Sci. USA **82**:4539–4543.

10. **Folks, T. M., D. Powell, M. Lightfoote, S. Koenig, A. S. Fauci, S. Benn, A. Rabson, D. Daugherty, H. E. Gendelman, M. D. Hoggan, et al.** 1986. Biological and biochemical characterization of a cloned Leu-3-cell surviving infection with the acquired immune deficiency syndrome retrovirus. J. Exp. Med. **164**:280–290.

11. **Furlini, G., M. C. Re, E. Ramazzotti, M. Vignoli, D. Zaccarelli, G. Zauli, and M. La Placa.** 1992. Absence of human immunodeficiency virus type 1 infection in blood donors with doubtful serum reactivity by Western blot analysis. Immunol. Infect. Dis. **2**:157–160.

12. **Gibellini, D., M. Zerbini, M. Musiani, S. Venturoli, G. Gentilomi, and M. La Placa.** 1993. Microplate capture hybridization of amplified parvovirus B19 DNA fragment labeled with digoxigenin. Mol. Cell. Probes **7**:101–106.

13. **Hsia, K., and S. A. Spector.** 1991. Human immunodeficiency virus DNA is present in a high percentage of CD4+ lymphocytes of seropositive individuals. J. Infect. Dis. **164**:470–475.

14. **Lafeuillade, A., C. Tamalet, P. Pellegrino, C. Tourres, N. Yahi, C. Vignoli, R. Quilichini, and P. de Micco.** 1993. High viral burden in lymph nodes during the early stage of HIV-1 infection. AIDS **7**:1527–1528.

15. **Lalli, E., D. Gibellini, S. Santi, and A. Facchini.** 1992. In situ hybridization in suspension and flow cytometry as a tool for the study of gene expression. Anal. Biochem. **207**:298–303.

16. **Levy, J. A.** 1993. HIV pathogenesis and long term survival. AIDS **7**:1401–1410.

17. **Nuovo, G. J., M. Margiotta, P. MacConnell, and J. Becker.** 1992. Rapid in situ detection of PCR amplified HIV-1 DNA. Diagn. Mol. Pathol. **2**:98–102.

18. **Ou, C. Y., S. Kwok, S. W. Mitchell, D. H. Mack, J. J. Sninsky, J. W. Krebs, P. Feorino, D. Warfield, and G. Schochetman.** 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science **239**:295–297.

19. **Pantaleo, G., C. Graziosi, L. Butini, P. A. Pizzo, S. M. Schnittman, D. P. Kotler, and A. S. Fauci.** 1991. Lymphoid organs function as major reservoirs for human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **88**:9838–9842.

20. **Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci.** 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature (London) **362**:355–358.

21. **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.

22. **Patterson, B. K., M. Till, P. Otto, C. Goolsby, M. R. Furtado, L. J. McBride, and S. M. Wolinsky.** 1993. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science **260**:976–979.

23. **Poznansky, M. C., B. Walker, W. A. Haseltine, J. Sodroski, and E. Langhoff.** 1991. A rapid method for quantitating the frequency of peripheral blood cells containing HIV-1 DNA. J. Acquired Immune Defic. Syndr. **4**:368–373.

24. **Re, M. C., G. Furlini, and M. La Placa.** 1989. Rapid detection of HIV-1 in clinical samples by co-culture with heat-shocked cells. J. Virol. Methods **26**:313–317.

25. **Rosenberg, Z. F., and A. S. Fauci.** 1989. The immunopathogenesis of HIV infection. Adv. Immunol. **47**:377–431.

26. **Saksela, K., C. Stevens, P. Rubinstein, and D. Baltimore.** 1994. Human immunodeficiency virus type 1 messenger RNA expression in peripheral blood cells predicts disease progression independently of the numbers of CD4+ lymphocytes. Proc. Natl. Acad. Sci. USA **91**:1104–1108.

27. **Schnittman, S. M., J. J. Greenhouse, M. C. Psallidopoulos, M. Baseler, N. P. Salzman, A. S. Fauci, and H. C. Lane.** 1990. Increasing viral burden in CD4+ T cells from patients with human immunodeficiency virus (HIV) infection reflects rapidly progressive immunosuppression and clinical disease. Ann. Intern. Med. **113**:438–443.

28. **Schnittman, S. M., M. C. Psallidopoulos, H. C. Lane, L. Thompson, M. Baseler, F. Massari, C. H. Fox, N. P. Salzman, and A. S. Fauci.** 1989. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. Science **245**:305–308.

29. **Simmonds, P., P. Balfe, J. F. Peutherer, C. A. Ludlam, J. O. Bishop, and A. J. Brown.** 1990. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J. Virol. **64**:864–872.

30. **Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40**:9–17.

31. **Yang, G., S. Garhwal, J. C. Olson, and G. N. Vyas.** 1994. Flow cytometric immunodetection of human immunodeficiency virus type 1 proviral DNA by heminested PCR and digoxigenin-labeled probes. Clin. Diagn. Lab. Immunol. **1**:26–31.