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Few Infected CD4$^+$ T Cells but a High Proportion of Replication-Competent Provirus Copies in Asymptomatic Human Immunodeficiency Virus Type 1 Infection

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The virus load in CD4$^+$ T cells from six asymptomatic human immunodeficiency virus type 1 (HIV-1)-infected individuals was determined by limiting-dilution analysis with a sensitive virus isolation procedure and the polymerase chain reaction (PCR). Both methods allowed detection of one HIV-1-infected cell among 10^5 uninfected cells. The number of provirus-containing CD4$^+$ T cells was found to be 1 per 4,000 to 150,000 (median, 1 per 29,000), as determined by virus isolation and 1 per 2,500 to 26,000 (median, 1 per 12,000), as determined by PCR. Infected cells contained an average of 1 to 2 provirus copies, and a high proportion of the provirus copies (1 in 1 to 1 in 6; median, 1 in 2) were replication competent. The results suggest that only a few CD4$^+$ T cells are likely to be lost as a direct consequence of the presence of HIV-1 in infected cells in asymptomatic individuals and that additional mechanisms may contribute to the depletion of CD4$^+$ T cells observed in vivo.

The most detrimental immunological consequence of infection with human immunodeficiency virus type 1 (HIV-1) is a depletion of CD4$^+$ T lymphocytes (19). Several mechanisms have been proposed to account for the loss of CD4$^+$ T cells. HIV-1 may exert a cytopathic effect on infected CD4$^+$ T cells, or infected cells may be killed by HIV-specific cytotoxic T cells or antibody-dependent cell-mediated cytotoxicity (19). Uninfected CD4$^+$ T cells may be involved in the formation of multinucleated giant cells; they may internalize and present HIV-1 antigens and as a result be killed by cytotoxic cells; or antilymphocyte autoantibodies may facilitate immune clearance of uninfected CD4$^+$ T cells (19). Reduced generation of new CD4$^+$ T cells may be caused by HIV-1 infection of bone marrow progenitor cells (7, 19) or thymic T-cell precursors (20).

The present study was initiated to answer questions pertaining to the cause and effect of the cytopathic effect exerted by HIV-1 on infected CD4$^+$ T cells. Accumulation of unintegrated viral DNA has been directly related to cytopathic effect in several nonhuman retroviral diseases (24). Could accumulation of HIV-1 DNA be detected in infected human CD4$^+$ T cells in vivo? Approximately 1% of the CD4$^+$ T cells of patients with AIDS have been found to contain HIV-1 proviral DNA. This high level of infection was proposed to be a primary cause for the low number of CD4$^+$ T cells in these patients (21). Could a similar proportion of infected CD4$^+$ T cells be found prior to the development of HIV-1-related disease? And how many of the HIV-1-infected cells contain replication-competent provirus copies?

We detected HIV-1 provirus in 1 per 2,500 to 26,000 (median, 1 per 12,000) recirculating CD4$^+$ T cells in asymptomatic HIV-1-infected individuals. A high proportion of infected cells contained replication-competent provirus copies. Each infected cell contained, on the average, less than two HIV-1 DNA copies.

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MATERIALS AND METHODS

Study participants. Six HIV-1-seropositive individuals were selected by the sole criterion that they have no symptoms or signs of disease (Centers for Disease Control [CDC] group II) (5). They all remained healthy during the course of this study. Subjects A, C, D, E, and F were Norwegian males. The duration of their infection was taken as the time from diagnosis of HIV-1 seropositivity when this was diagnosed after the first HIV-1 antibody test performed (subjects A, D, and E, Table 1) or as the time from a date midway between the last negative and the first positive HIV-1 antibody test (subjects C and F, Table 1). Subject B, a man of African origin, was tested for the first time immediately prior to this investigation. The approximate time of onset of his infection could not be determined. Blood was sampled on two occasions 3 to 4 months apart for four of the participants, and for subject F a third sample was obtained 11 months after the first test. CD4$^+$ and CD8$^+$ T cells were counted directly in blood by an immunomagnetic method (3); the results were well within the range observed for individuals in CDC group II (Table 1) (4). Blood from HIV-seronegative donors was obtained from the Red Cross Blood Bank, Oslo.

MAb and immunomagnetic beads. Monoclonal antibodies (MAb) specific for CD4 (clone 66.1), a generous gift from J. Hansen, Fred Hutchinson Cancer Center, Seattle, Wash.) and a monomorphic epitope of the T-cell receptor αβ heterodimer (clone T10/B9; a generous gift from J. S. Thompson, University of Kentucky Medical Center, Lexington, Ky.) were coated onto immunomagnetic beads (Dynabeads M-450; Dynal, Oslo, Norway) at 4 μg/mg of Dynabeads as described previously (8).

Preparation of cells. Peripheral blood mononuclear cells (PBMC) were obtained from citrate-anticoagulated blood by Isopaque-Ficoll gradient centrifugation, washed twice in phosphate-buffered saline (PBS), and finally resuspended in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum (FCS) and antibiotics (RPMI-FCS). CD4$^+$ T cells were isolated as described previously (2). Briefly, Dynabeads

TABLE 1. Concentration of $CD4^+$ and $CD8^+$ T cells in blood and duration of infection

Subject	Test no.[a]	No. of cells (10^9/liter)		Duration of infection (yr)[b]
		CD4	CD8	
A	1	0.58	0.87	4
	2	0.67	1.05	
B	1	0.40	0.56	ND[c]
C	1	ND	ND	2
	2	0.76	1.39	
D	1	0.59	0.69	4
E	1	0.75	1.53	1
	2	0.70	1.54	
F	1	0.51	1.15	1
	2	0.52	1.08	
	3	0.36	1.35	2
CDC group II[d] (n = 120)		0.54 (0.17–2.04)	0.88 (0.22–2.19)	

[a] Tests 1 and 2 were performed 3 to 4 months apart; test 3 (subject F) was performed 11 months after test 1.
[b] For estimation of duration of infection, see Materials and Methods.
[c] ND, Not determined.
[d] Median values and ranges (in parentheses) for the number of $CD4^+$ and $CD8^+$ T cells in blood for 120 HIV-1-infected individuals in CDC group II are included for comparison (4).

coated with anti-CD4 MAb 66.1 (10 beads per target cell) were incubated with PBMC at 4 to 6°C for 1 h. Rosetted cells were isolated by the application of a magnet (magnetic particle separator; Dynal) to the side of the test tube, washed three times in cold PBS with 2% (vol/vol) FCS, and subsequently resuspended in RPMI-FCS. Cells and beads were separated by overnight incubation at 37°C, followed by resuspension by moderate pipetting and isolation of the beads to the side of the test tube with the magnet. Nonrosetted cells were transferred to another test tube and incubated for another 24 h at 37°C. At this time, the cells were either activated in cell culture or frozen at −70°C in 10% dimethyl sulfoxide for PCR studies. $CD4^+$ T cells isolated in this way contain <2% contaminating $CD8^+$ T cells (flow cytometric studies) and show >95% viability, as determined by acridine orange-ethidium bromide staining.

Cell cultures. Cultures for virus isolation were established by a modification of a procedure described previously (2). Serial fivefold dilutions of patient $CD4^+$ T cells (10^5, 2×10^4, 4×10^3, 8×10^2, and 1.6×10^2 cells per well), usually 10 parallel wells at each dilution, were cocultured with $CD4^+$ T cells from HIV-seronegative blood bank donors (10^5 cells per well). The cells were activated with Dynabeads coated with anti-T-cell receptor MAb T10/B9 (four beads per cell) and cultured in RPMI-FCS supplemented with 10 U of recombinant interleukin-2 (Amersham International, Amersham, England) per ml. The culture medium was changed twice a week, and the supernatants were screened for the presence of HIV p24 by antigen capture enzyme-linked immunosorbent assay (Abbott) on day 18. Control cultures of $CD4^+$ T cells from the HIV-seronegative donors at 10^6 cells per well were run in parallel. The HIV-1-infected cell line ACH-2 (6) (provided by the AIDS Research and Reference Reagent Program; contributed to the program by T. Folks) was maintained unstimulated in culture in RPMI-FCS.

PCR. PCR was performed by a nested primer method which was described recently (1). The *pol* gene-specific primer pairs JA17 plus JA20 and JA18 plus JA19, which have been shown to detect 99% of a wide variety of clinical isolates (1), were used in this study. Materials for PCR was prepared in two different ways: by dilution of the $CD4^+$ T cells prior to cell lysis and by dilution of DNA from the $CD4^+$ T cells after cell lysis. In either case, the cells were lysed in PCR lysis buffer (10 mM Tris-HCl [pH 8.3], 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Tween, 300 μg of proteinase K per ml), and amplification was performed on 10-μl aliquots containing 1×10^5, 2×10^4, 4×10^3, 8×10^2, or 1.6×10^2 $CD4^+$ T cells or cell equivalents of DNA. The sensitivity of the PCR procedure was determined by amplification of aliquots of cell lysates containing DNA corresponding to five ACH-2 cells and 10^5 HIV-seronegative PBMC and to 0.5 ACH-2 cell and 10^5 PBMC.

PCR was also performed on cells from some of the $CD4^+$ T-cell limiting-dilution coculture wells. For these experiments, all the cells in the wells were frozen on day 21 of the culture and stored at −70°C. After being thawed, the cells were counted and lysed as described above. HIV-1 DNA was detected by amplification of 10-μl aliquots containing DNA corresponding to 10^5 cells. The number of HIV-1 DNA copies per culture well was determined by limiting-dilution analysis. Control PCR was performed on aliquots of 10^5 $CD4^+$ T cells from seronegative donors and on lysis buffer without cells.

Statistical considerations. The frequency of HIV-1 provirus-containing cells was determined by limiting-dilution analysis and statistical methods originally designed to determine the precursor frequency of antigen-specific T cells (26). According to the Poisson distribution formula, the precursor frequency is the inverse fraction of the number of cells required to give 63% positive wells or reactions.

RESULTS

Sensitivity and specificity of PCR and virus isolation. The sensitivity of the PCR was tested by using ACH-2 cells, which contain one HIV-1 provirus copy per cell (6). When PCR was performed on aliquots containing DNA corresponding to 0.5 ACH-2 cell and 10^5 PBMC from an HIV-1-seronegative donor, 4 of 10 reactions were positive for HIV-1 DNA (Fig. 1). Thus, PCR detected 1 copy of HIV-1 among 10^5 cells. Similar results could not be obtained by virus isolation, possibly because the production of virus by each ACH-2 cell induced by phorbol ester myristate is very low, several orders of magnitude lower than the production of virus by each infected primary $CD4^+$ T lymphocyte (1a, 2, 16). Specificity was tested by performing PCR on 10^5 $CD4^+$ T cells (n = 17 from 10 different donors) and virus isolation from 10^6 $CD4^+$ T cells from the 10 HIV-1-seronegative donors of the allogeneic coculture cells. These were all negative.

Proportion of HIV-1 provirus-containing cells. The proportion of $CD4^+$ T cells containing HIV-1 proviral DNA and the proportion containing replication-competent provirus copies was determined by PCR and virus isolation, respectively, on serial fivefold dilutions of $CD4^+$ T cells (Table 2). The proportion of HIV-1 provirus-containing cells by either method varied considerably among the study participants. However, analyses of two or three samples collected 3 to 11 months apart from four of the participants gave very similar

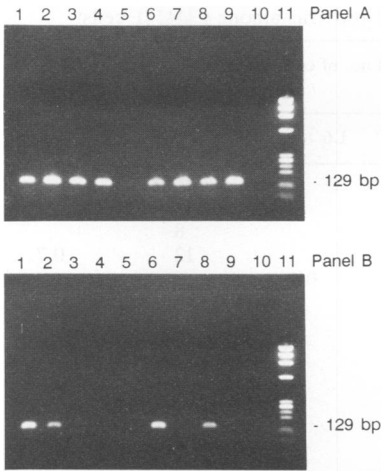

FIG. 1. Detection of HIV-1 provirus DNA in ACH-2 cells by PCR with nested primers. PCR was performed on aliquots containing DNA corresponding to five ACH-2 cells and 10^5 PBMC from a healthy blood donor (A, lanes 1 to 4 and 6 to 9) and on aliquots containing DNA corresponding to 0.5 ACH-2 cell and 10^5 PBMC (B, lanes 1 to 10). Lanes 5 and 10 (panel A) represent PCR performed on the lysis buffer only (negative controls). Lanes 11 (panels A and B) contain DNA size markers (*Hae*III digest of φX174 RF DNA). The expected size of the amplified *pol* gene product (129 bp) is indicated to the right on both panels.

results, demonstrating the reproducibility of the determinations for clinically stable individuals. The proportion of $CD4^+$ T cells containing replication-competent HIV-1 provirus copies ranged from 1 per 4,000 to 1 per 150,000 (median, 1 per 29,000) cells, while the proportion of $CD4^+$ T cells containing HIV-1 proviral DNA ranged from 1 per 2,500 to 1 per 26,000 (median, 1 per 12,000) cells. In subjects A, E, and F, more than half of the provirus copies were replication competent, while subjects C and D had six and five proviral copies per replication-competent copy, respectively.

In test 2 on subjects E and F, the results were controlled by performing PCR on cells from the virus isolation wells containing 2×10^4 and 4×10^3 patient cells (subject E) and 8×10^2 patient cells (subject F). All the virus isolation-positive wells were also positive by PCR. In addition, four wells (subject E, 2×10^4 cells) and two wells (subject F, 8×10^2 cells) were positive by PCR. All the wells containing 4×10^3 $CD4^+$ T cells from subject E were negative by PCR. These results were very similar to the results obtained by performing PCR on uncultured cells (Table 2). The wells which were virus isolation positive and PCR positive contained >1,000 HIV-1 DNA copies. In contrast, the virus isolation-negative but PCR-positive wells contained <15 HIV-1 DNA copies, suggesting multiplication by duplication during cell division rather than by virus replication and infection of new cells. The total number of cells in these culture wells increased from 1×10^5 to approximately 7×10^5 to 8×10^5.

Number of HIV-1 DNA copies per infected cell. In order to determine the average number of HIV-1 DNA copies contained in each infected cell, DNA was prepared from $CD4^+$ T cells and subsequently diluted so that PCR was performed on the same amount of DNA as in the equivalent dilutions of whole cells. The number of $CD4^+$ T-cell equivalents of DNA required for positive PCR was 1,200 to 19,000 (median, 11,000) (Table 2). The average number of HIV-1 DNA copies per infected cell was calculated by dividing the fraction of $CD4^+$ T-cell DNA equivalents containing one molecule of HIV-1 by the fraction of whole $CD4^+$ T cells containing one HIV-1-infected cell by PCR. The results show that each infected cell contained an average of one to two HIV-1 DNA copies (Table 2).

DISCUSSION

We used virus isolation and PCR with nested primers in limiting-dilution analysis of various aspects of viral load in $CD4^+$ T cells in the asymptomatic phase of HIV-1 infection. The methods used were sensitive, specific, and reproducible. One infected cell, containing one HIV-1 provirus copy (6), was detected by PCR among 10^5 uninfected cells. In two of the subjects (A and F), the proportion of cells containing HIV-1 provirus was reproducibly similar whether PCR or virus isolation was used. This shows that the virus isolation method was also reproducibly able to detect one cell infected with a replication-competent provirus copy among 10^5 uninfected cells.

The proportion of $CD4^+$ T cells infected with HIV-1 in these asymptomatic subjects was low, ranging from 1 in 2,500 to 1 in 26.000. Our results are consistent with those reported by others from studies of asymptomatic HIV-1-infected individuals (10, 17, 23) but contrast with the recent observation that in patients with AIDS, 1 in 100 $CD4^+$ T cells contains HIV-1 provirus (21). Although we show that a high proportion of the infected cells contain replication-competent provirus copies, it is difficult to accept that a cytopathic effect by HIV-1 on individual infected cells can kill sufficient numbers of $CD4^+$ T cells to exceed the capacity for generation of new $CD4^+$ T cells if this remains normal (13). It could be argued that recirculating $CD4^+$ T cells may not be representative of the total pool of $CD4^+$ T cells. However, lymph nodes were recently shown to contain an average of only 0.001 or fewer HIV-1 DNA copies per cell in most individuals studied (clinical status not given) (22). A considerably lower proportion of lymph node cells have been found to contain HIV-1 RNA (9), and the cells containing HIV-1 RNA may be predominantly follicular dendritic cells (25). Thus, while a low-grade persistent replication of HIV-1 is likely to occur (10, 25), there is no evidence of sequestration and destruction of large numbers of HIV-1-infected $CD4^+$ T cells in lymph nodes.

Recent studies of patients with HIV-1-related disease or AIDS have suggested that most HIV-1-infected cells contain replication-incompetent provirus copies (14, 21). It was proposed that defective genomes may be responsible for modulation of immune responses and pathogenesis of HIV-1-related disease (14). The isolation of a highly defective strain of HIV-1 from a healthy Gabonese individual, on the other hand, was taken to suggest that the presence of predominantly defective genomes might be associated with maintenance of health (11). We show that in the asymptomatic HIV-1-infected participants in this study, a high proportion of the provirus copies were replication competent and that this was fully compatible with good health and a high number of $CD4^+$ T cells in the blood even after 4 years of infection with HIV-1.

There was no evidence of accumulation of HIV-1 DNA in the recirculating $CD4^+$ T cells in this study. Similar observations have been made for PBMC from asymptomatic individuals and $CD4^+$ T cells from patients with AIDS (21, 23). A low degree of accumulation of HIV-1 DNA can be

TABLE 2. Limiting-dilution analysis of the occurrence of HIV-1 provirus among CD4$^+$ T cells[a]

Subject	Test no.	Method	Material diluted	No. of positive tests/no. performed at indicated no. of cells or cell DNA equivalents					No. of cells (10^3) per HIV copy	No. of DNA copies per functional copy	No. of DNA copies per cell
				1×10^5	2×10^4	4×10^3	8×10^2	1.6×10^2			
A	1	VI	Cells	10/10	8/10	3/10			14		
	2	VI	Cells	10/10	9/10	7/10	0/10		8		
		PCR	Cells	7/7	6/10	6/10	1/10		12	0.7	
		PCR	DNA		10/10	5/10	3/10	0/4	6		2.0
B	1	VI	Cells	10/10	6/10	1/10			22		
C	1	VI	Cells	6/10	1/10				108		
	2	VI	Cells	5/10	0/10				150		
		PCR	Cells	6/6	6/10	1/10	1/10		24	6.2	
		PCR	DNA		7/10	2/10	1/10		19		1.3
D	1	VI	Cells	9/10	4/10	2/10	0/5		36		
		PCR	Cells		5/5	2/5	0/5		7	5.1	
E	1	VI	Cells	9/10	2/10	1/10			50		
		PCR	Cells	8/8	4/9	2/9	0/9		26	1.9	
		PCR	DNA		9/12	0/12	0/12		16		1.6
	2	VI	Cells	8/10	4/10	0/10			50		
		PCR	Cells	6/9	7/9	4/9	1/10		25	2.0	
		PCR	DNA		7/9	0/9	0/9	0/9	14		1.8
F	1	VI	Cells	10/10	10/10	3/10	2/10	0/5	7		
		PCR	Cells	8/8	10/10	6/10	1/10	0/10	5	1.4	
		PCR	DNA		12/14	8/14	1/14	0/15	8		0.6
	2	VI	Cells		10/10	7/10	2/10	0/5	4		
		PCR	Cells	6/6	8/8	4/10	3/9	2/10	5	0.8	
		PCR	DNA		8/9	4/9	6/9	0/10	4		1.3
	3	VI	Cells		10/10	7/10	2/10	0/5	4		
		PCR	Cells		10/10	10/10	5/10	0/10	2.5	1.6	
		PCR	DNA		10/10	10/10	8/10	1/10	1.2		2.0

[a] Limiting-dilution analysis was performed on serial fivefold dilutions of CD4$^+$ T cells by virus isolation (VI) and PCR and on serial dilutions of CD4$^+$ T-cell DNA corresponding to equivalent numbers of cells by PCR. The number of cells per HIV-1 copy was determined according to the Poisson distribution formula to be the number of cells or cell equivalents of DNA required to give 63% HIV-1-positive wells or reactions. The number of DNA copies per functional copy was found by dividing the fraction of CD4$^+$ T cells containing one HIV-1 provirus copy as determined by PCR by the fraction containing one provirus copy as determined by virus isolation. The number of DNA copies per cell was found by dividing the fraction of CD4$^+$ T-cell equivalents of DNA containing one provirus copy of HIV-1 by the fraction of whole CD4$^+$ T cells containing one infected cell as determined by PCR.

observed following acute infection of CD4$^+$ T cells in vitro (12), while cytopathogenically important accumulation of HIV-1 DNA can be found only as a result of multiple infection events in vitro (15, 18). Similar observations have been made for nonhuman retroviruses (24). Our results suggest that HIV-1-infected cells within the recirculating pool of CD4$^+$ T cells are generally not acutely infected or exposed to multiple infection events. The low occurrence of HIV-1 DNA in lymph nodes from HIV-1-infected individuals also indicates that this cytopathogenic effector mechanism is unlikely to make a major contribution to the depletion of CD4$^+$ T cells (22).

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