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Detection of CD4$^+$ T Cells Harboring Human Immunodeficiency Virus Type 1 DNA by Flow Cytometry Using Simultaneous Immunophenotyping and PCR-Driven In Situ Hybridization: Evidence of Epitope Masking of the CD4 Cell Surface Molecule In Vivo

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Human immunodeficiency virus type 1 (HIV-1) infection of T cells and cells of the monocyte/macrophage lineage requires a specific interaction between the CD4 antigen expressed on the cell surface and the HIV-1 external envelope glycoprotein (gp120). To study the association between HIV-1 infection and modulation of cell surface expression of the CD4 molecule in vivo, we examined the CD4$^+$ T cells harboring proviral DNA obtained from HIV-1-infected individuals who had received no antiretroviral therapy for at least 90 days. Simultaneous immunophenotyping of CD4 cell surface expression and PCR-driven in situ hybridization for HIV-1 DNA were used to resolve the CD4$^+$ T cells into distinct populations predicted upon the presence or absence of proviral DNA. Among the HIV-1-infected study subjects, the percentage of CD4$^+$ T cells harboring proviral DNA ranged from 17.3 to 55.5%, with a mean of 40.5%. Cell surface fluorescent staining with anti-CD4 antibody directed against a non-gp120 binding site-related epitope (L120) or a conformation-dependent epitope of the gp120 binding site (Leu 3A) demonstrated either an equivalent or a 1.5- to 3-fold-lower cell surface staining intensity for the HIV-1 DNA-positive subpopulation relative to the HIV-1 DNA-negative subpopulation, respectively. These data suggest that masking or alteration of specific epitopes on the CD4 molecule occurs after viral infection.

The persistent viral replication that follows human immunodeficiency virus type 1 (HIV-1) infection during the period of clinical quiescence is associated with aberrations in T-cell function (5, 30, 52) and either a stable, slowly declining, or precipitously declining CD4$^+$ T-cell count (43). The selective depletion of CD4$^+$ T cells may be a consequence of a number of potential immunopathogenic mechanisms. In addition to direct HIV-1-induced cytopathicity, several indirect mechanisms, including syncytium formation (24, 47), free gp120/gp160-mediated cell killing (33, 46), antibody-dependent cellular cytotoxicity (22, 51), autoimmune reactions (34), and apoptosis mediated by CD4 cross-linking (15), have been invoked to explain CD4$^+$ T-cell depletion in association with low-level virus replication. Recent estimates of high-level plasma-free RNA (37) and cell-associated DNA in blood (1, 36) and lymphoid tissue (8, 11, 34) support a direct role for viral replication in disease pathogenesis.

Qualitative abnormalities of T-cell function are characterized by a selective inability to proliferate after self-major histocompatibility complex (MHC) class II-restricted antigen stimulation in vitro (39). The mechanism by which HIV-1 perturbs T-cell function is not understood. High-affinity binding of gp120 to CD4 may impede the usual interaction of CD4 with class II major histocompatibility complex molecules on the surface of antigen-presenting cells (7, 13, 28, 42). Alternatively, gp120-CD4 binding may interfere with the T cell-specific cytoplasmic tyrosine protein kinase p56^{lck}-mediated signal transduction that follows ligand binding to the CD4 molecule (14, 45) or lead to specific cytokine secretion (4, 10, 18, 38).

HIV-1 infection may also decrease the cell surface expression of CD4 (19, 41, 48) and other molecules involved in the T-cell response (25). CD4 cell surface expression may be reduced by modulation of CD4 transcription (17, 49) or by sequestration of CD4–HIV-1 glycoprotein precursor (gp160)–p56^{lck} complex in the endoplasmic reticulum (6). Alteration of the cell surface CD4 molecule has a net effect of causing T-cell receptor (TCR) perturbation and T-cell dysfunction (3, 23). Discerning the potential immunopathogenic mechanisms by which HIV-1 perturbs T-cell function in vivo has been hampered by the technical inability to simultaneously study the CD4 surface molecule and the viral genetic material at a single-cell level.

To determine the number of CD4$^+$ T cells within a heterogenous cell population that harbor the virus, we performed simultaneous intracellular HIV-1 amplification and cell surface immunophenotyping. Thirteen HIV-1-infected study participants and seven uninfected controls were evaluated for intracellular DNA and CD4 surface staining by PCR-driven in situ hybridization and flow cytometry. To screen for potential changes in the CD4 surface molecule on infected cells, a subset of HIV-1-infected patients was also examined for altered bind-

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ing of anti-CD4 to potential gp120-occluded and nonoccluded determinants on CD4. Our results show that a significant proportion (17.3 to 55.5%) of $CD4^+$ T cells in blood contain HIV-1 DNA. Additionally, we found a marked disparity in CD4 cell surface staining between HIV-1-infected and uninfected cell populations, which is consistent with cell surface CD4 epitope masking. The latter observation could have implications for monitoring disease progression.

MATERIALS AND METHODS

Study subjects. Thirteen participants with documented HIV-1 infection and seven uninfected participants from the Chicago component of the Multicenter AIDS Cohort Study were selected for this study. All 13 HIV-1-infected subjects had not received any antiretroviral therapy for at least 90 days preceding venous blood sampling. Each participant had a relatively stable $CD4^+$ T-cell number for at least 18 months preceding blood sampling. All analyses were performed in a blinded fashion with respect to the HIV-1 infection status of the study subjects. Informed consent was obtained from all participants before enrollment into this study.

Cells and cell lines. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood layered on a Histopaque 1077 (Sigma, St. Louis, Mo.) discontinuous density gradient and centrifuged at 600 × *g* for 30 min at ambient temperature. The turbid layer was removed and was washed twice with 3 volumes of RPMI medium and once with phosphate-buffered saline (PBS). Monocytes were removed with unconjugated CD14 (Becton-Dickinson, San Jose, Calif.) and anti-human immunoglobulin-coated magnetic beads (Dynal, Great Neck, N.Y.) according to the manufacturer's protocol. The 8E5/LAV cell line (AIDS Research and Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.), containing a single copy of integrated HIV-1 proviral DNA per cell, was harvested at an early passage and used as the HIV-1-infected cell copy number control. Uninfected CEM cells (AIDS Research and Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and CEM cells productively infected with the HIV-1 NL4-3 strain at an early passage were used as negative and positive controls, respectively.

PCR-driven in situ hybridization. Cell samples were adjusted to a final concentration of 10^6 cells per ml. A 40-μl aliquot of each sample was centrifuged at 600 × *g* for 2 min at ambient temperature. The supernatant was removed, and the cell pellet was resuspended in 90 μl of PBS and 10 μl of biotinylated anti-CD4 (Becton-Dickinson). Cells were again centrifuged at 300 to 600 × *g* for 2 min, and the cell pellet was washed twice in PBS. The cells were then fixed and permeabilized by the addition of 50 μl of Permafix (Ortho Diagnostics, Inc., Raritan, N.J.) at ambient temperature for 60 min. Cells were then pelleted as above, washed with PBS, and resuspended in 190 μl of a PCR mixture consisting of 10 mM Tris HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.25 mM (each) dATP, dCTP, and dGTP; 0.14 mM dTTP; 4.3 μM dUTP-11-digoxigenin; 100 pmol (each) of forward and reverse primers; 1.0 μl (5 U) of *Taq* polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, Conn.); and gelatin (0.001%, wt/vol). The DNA in the reaction mixture was amplified in 500-μl tubes inserted into the wells of a 48-well thermocycler (Perkin-Elmer Cetus) programmed for 25 cycles of thermal denaturation (94°C, 1 min), primer annealing (58°C, 2 min), and primer extension (74°C, 1.5 min), with 5 s added for each of the 25 cycles. Appropriate positive and negative controls amplified with or without the addition of *Taq* polymerase were simultaneously run with each sample.

After in vitro amplification, the cells were pelleted and resuspended in 25 μl of 10 mM Tris HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$. A 100-ng aliquot of the appropriately labeled target-specific oligonucleotide probe in 10 μg of sonicated herring sperm DNA (Sigma) per ml was added to the reaction tube. The product DNA was denatured at 95°C for 3 min and then allowed to hybridize with the respective oligonucleotide probe at 56°C for 2 h. After hybridization, the cells were washed for 30 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–500 μg of bovine serum albumin (BSA) per ml at 42°C, 30 min with 1× SSC–50% formamide–500 μg of BSA per ml at 42°C, 30 min with 1× SSC–5 μg of BSA per ml at ambient temperature, and then briefly with PBS at ambient temperature.

Flow cytometry. After the last wash, the cells were resuspended in 80 μl of PBS and 20 μl of streptavidin-phycoerythrin (PE) and incubated for 30 min at ambient temperature. The cells were then washed in PBS as described above. The cell suspension was filtered through a 37-μm-pore-size nylon mesh and analyzed by flow cytometry, using an EPICS PROFILE III flow cytometer. Laser excitation was 15 mW at 488 nm, and the fluorescein isothiocyanate and PE fluorescence were detected with a standard optical filter setup (550 dichroic, 525 bandpass [fluorescein isothiocyanate], and 585 bandpass [PE]). Instrument sensitivity was standardized before each experiment, employing Immuno-Bright calibration beads (Coulter Source, Marietta, Ga.). The percent fluorescence-positive cells was determined by integration over a range of 0.2% positive counts on the identically treated negative sample (100% uninfected PBMCs).

Probes and primers. Sequence-specific oligonucleotide probes (Applied Biosystems, San Diego, Calif.) containing 5′- and 3′-labeled 5-carboxyfluorescein were synthesized on an Applied Biosystems 380B DNA synthesizer with 5′-carboxyfluorescein phosphoramidite (36). The synthesized material was alkaline deprotected and purified by high-performance liquid chromatography. 5′-Carboxyfluorescein phosphoramidite incorporation was verified by UV spectroscopy. HIV-1 *gag*-specific primers SK39 and G51 (Becton-Dickinson) and HLA-DQα-specific primers GH26 and GH27 were used as target sequence-specific and control primers, respectively (27).

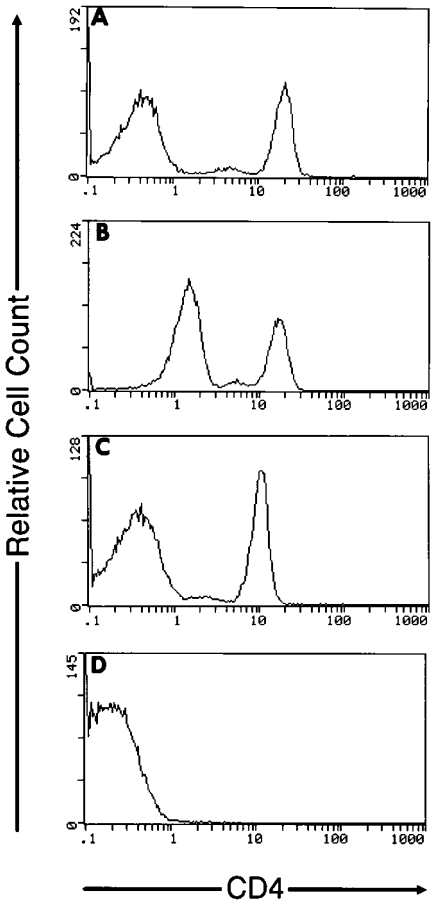

FIG. 1. Effectiveness of various biotinylated anti-CD4 antibody-binding schemes on cells subjected to thermal amplification in situ. Curves were generated using cells with anti-CD4 antibody bound before fixation, permeabilization, and detection by streptavidin-PE (A), cells with anti-CD4 antibody bound after fixation, permeabilization, and detection without thermal amplification (B), and cells with anti-CD4 antibody bound before fixation and permeabilization, followed by thermal amplification (C). For panel C, streptavidin-PE was added after thermal amplification and hybridization. The curve in panel D was generated using cells with anti-CD4 antibody bound after fixation, permeabilization, and thermal amplification. Abscissa, log fluorescence.

Antibodies. Immunophenotyping was performed with antibody concentrations and protocols recommended by the manufacturers. The antibodies used included biotinylated anti-CD5 (Leu 1), anti-CD4 (Leu 3A), L120, and unconjugated anti-CD14 (Leu M3) (Becton-Dickinson). Fluorescein isothiocyanate-conjugated anti-CD4 (Caltag, San Francisco, Calif.) and PE-conjugated anti-CD4 (Coulter, Hialeah, Fla.) were used in experiments to determine antibody stability during thermal cycling. Comparison of the relative intensities of cell surface staining was done by determining the mean peak fluorescence of the selected groups. CG-10, an antibody which binds to CD4-gp120 complexes, was used for cell surface marker staining and was generously provided by Jonathan Gershoni of Tel Aviv University.

Statistical analysis. Statistical analysis of pre- and postcycling determinations of cell surface markers was performed with the chi-square test; comparison of mean peak fluorescence differences was performed with paired *t* tests.

RESULTS

Determination of selective cell loss before and after thermal cycling. To determine the percentages of specific subpopula-

TABLE 1. PCR-driven in situ hybridization and immunophenotyping results[a]

Patient no.	$CD4^+$ T-cell count	% CD4 T cells	HIV-1 DNA-positive cells		Mean peak fluorescence (Leu 3A-PE) of $CD4^+$ T cells which are[b]:	
			% of PBMCs[c]	% of $CD4^+$ T cells	HIV^-[d]	HIV^+
1	11	19.2 ± 2.3	8.1 ± 2.1	42.1	2.7 ± 0.2	1.8 ± 0.2
2	20	23.6 ± 1.4	4.1 ± 0.6	17.3	2.9 ± 0.2	2.1 ± 0.2
3	85	9.2 ± 0.6	4.2 ± 0.9	45.6	6.8 ± 0.4	3.8 ± 0.2
4	98	22.3 ± 1.6	5.0 ± 1.2	22.4	8.5 ± 0.1	3.8 ± 0.2
5	259	27.7 ± 0.4	14.2 ± 1.6	51.2	2.8 ± 0.1	2.0 ± 0.1
6	263	19.0 ± 0.7	8.4 ± 1.4	44.2	9.2 ± 0.1	3.7 ± 0.2
7	372	28.1 ± 1.2	15.6 ± 0.5	55.5	9.1 ± 0.6	6.2 ± 0.1
8	420	26.5 ± 1.5	11.5 ± 1.1	43.3	5.5 ± 0.3	2.2 ± 0.2
9	506	27.1 ± 1.2	11.0 ± 1.3	40.5	6.1 ± 0.2	4.7 ± 0.7
10	513	19.0 ± 1.1	3.6 ± 0.5	18.9	8.8 ± 0.4	6.1 ± 0.2
11	530	31.1 ± 1.3	10.4 ± 0.7	33.2	9.8 ± 0.5	5.6 ± 0.3
12	742	30.4 ± 0.4	10.1 ± 2.7	33.2	9.6 ± 0.5	4.6 ± 0.5
13	1,431	39.3 ± 0.7	14.9 ± 0.8	37.9	12.4 ± 0.2	4.5 ± 0.5
14	1,046	35.7 ± 1.2	0	0	11.2 ± 0.4	0

[a] Flow cytometric analysis of cells isolated from HIV-1-infected subjects with stable $CD4^+$ T-cell counts who had not received any antiretroviral therapy for at least 90 days preceding venous blood sampling

[b] Fluorescence is expressed in fluorescence units.

[c] Monocyte depleted.

[d] Mean peak fluorescence of Leu 3A staining on $CD4^+$ T cells from seven additional uninfected controls ranged from 2.3 to 11.2.

tions of cells and to monitor possible selective loss of certain subpopulations of cells during thermal cycling, we used fluorescein-conjugated monoclonal antibodies specific for T-cell antigens for immunophenotyping. Magnetic-bead enrichment of lymphocytes was performed following Ficoll-Hypaque discontinuous density gradient separation of PBMCs to ensure the absence of monocytes in populations of cells with decreased CD4 (Leu 3A) expression. Negative sorting with anti-CD14 antibody resulted in at least 98% lymphocytes, with less than 2% monocytes in all samples. The precycling percentage of cells expressing the CD4 (L120 and Leu 3A) and CD5 cell surface antigens following enrichment did not differ from the postcycling percentage of cells expressing these antigens ($P > 0.2$ for all χ^2 tests).

Effects of thermal cycling on immunophenotyping. A variety of antibody conjugations was used in immunophenotyping experiments to determine resiliency during thermal cycling. Biotinylated anti-CD4 antibody was bound to cells before fixation and permeabilization without subsequent thermal cycling (Fig. 1A); after fixation and permeabilization with subsequent thermal cycling (Fig. 1B); before fixation and permeabilization with subsequent thermal cycling (Fig. 1C); and after fixation, permeabilization, and thermal cycling (Fig. 1D). Biotin-conjugated anti-CD4 bound before fixation and permeabilization with subsequent thermal cycling (Fig. 1C) produced results comparable to those for the uncycled controls (Fig. 1A). Direct PE-conjugated antibodies lost fluorescence following thermal cycling, and direct fluorescein isothiocyanate-conjugated anti-CD4 antibody bound nonspecifically to >90% of cells when bound before or after thermal cycling (data not shown). Biotin- and PE-conjugated anti-CD4 antibodies added after thermal cycling failed to detect cells expressing the CD4 cell surface antigen. On the basis of these results, simultaneous immunophenotyping and in situ hybridization were performed with biotin-conjugated anti-CD4 antibodies that were bound to the cell surface before fixation and thermal cycling. This antibody labeling scheme ensures that cell surface marker determinations on cycled cells can be extrapolated to uncycled cells.

Quantification of $CD4^+$ cells containing HIV-1 proviral DNA. Quantification of $CD4^+$ T cells harboring proviral DNA was determined by PCR-driven in situ hybridization and two-color flow cytometry, using simultaneous immunophenotyping with the anti-CD4 antibody Leu 3A and with HIV-1 *gag*-specific primer pairs and a fluorescein-labeled target-specific probe. The percentage of $CD4^+$ (Table 1) T cells was determined following monocyte depletion, in order to restrict our analysis to $CD4^+$ T cells. The percentage of $CD4^+$ T cells harboring HIV-1 proviral DNA ranged from 17.3 to 55.5% (Table 1) with a median of 40.5%, while the percentage of infected PBMCs ranged from 3.6 to 15.6%, with a median of 10.1%. Negative controls consisting of PBMCs, isolated from a donor not infected with HIV-1, amplified and probed with HIV-1-specific oligonucleotides; HIV-1-infected 8E5 cells and PBMCs, isolated from an HIV-1-infected donor, amplified with HIV-1-specific primers and probed with an internally conserved oligonucleotide probe without *Taq* polymerase; and HIV-1-positive cells and PBMCs amplified with HLA-DQα primers and probed with an HIV-1-specific oligonucleotide probe all lacked HIV-1-positive cell subpopulations. The percentage of infected PBMCs or $CD4^+$ T cells did not correlate with $CD4^+$ T-cell count or with the percent $CD4^+$ T cells in these patients.

Determination of CD4 modulation in cells containing HIV-1 DNA. To determine the association of HIV-1 infection with CD4 conformation and cell surface expression on cells containing HIV-1 proviral DNA, we used PCR-driven in situ hybridization and two-color flow cytometry with a panel of antibodies directed at multiple epitopes. A conformation epitope of CD4 associated with the gp120-CD4 binding site was screened by Leu 3A, the prototype anti-CD4 antibody in commercial use. An epitope in CD4 domain 4 distal from the HIV-1 gp120 binding site was screened, using L120. To determine if there was any selective loss of T-cell subsets, an antibody directed against the pan-T-cell marker CD5 was used.

The total numbers of $CD4^+$ T cells in a given blood sample were comparable, regardless of whether they were enumerated before thermal cycling or after thermal cycling with or without DNA amplification and resolution into specific CD4-positive, HIV-1 proviral DNA-positive or CD4-positive, HIV-1 proviral DNA-negative subpopulations. These results indicate that

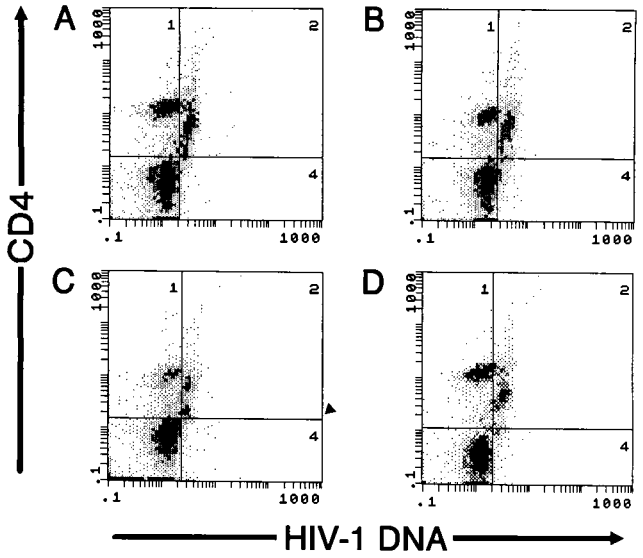

FIG. 2. Comparison of representative two-color dot plots of monocyte-depleted PBMCs from patients with varying $CD4^+$ T cell counts. Simultaneous immunophenotyping and PCR-driven in situ hybridization were performed on monocyte-depleted PBMCs as described, using the anti-CD4 antibody, Leu 3A. The double-positive (CD4-positive, HIV-1 DNA-positive) populations for each sample are in quadrant 2 of each plot. The patient numbers, which correspond to those in Table 1, and $CD4^+$ T-cell counts are as follows: (A) patient 13, 1,431; (B) patient 12, 742; (C) patient 6, 263; and (D) patient 9, 506. Ordinate, log red fluorescence. Abscissa, log green fluorescence.

there was no selective loss of either of these resolved T-cell subpopulations. Additionally, among these two T-cell subpopulations in each of the 13 tested HIV-1-positive clinical samples, the mean peak fluorescence of Leu 3A-stained cells was consistently decreased by 1.5- to 3-fold in the CD4-positive, HIV-1 proviral DNA-positive population when compared with the CD4-positive, HIV-1 proviral DNA-negative cell population (Table 1 and Fig. 2). In 12 of the 13 patient samples, the CD4-positive, HIV-1 proviral DNA-positive T cells appeared as a single population with a decreased mean peak fluorescence. In one sample (Fig. 2C [patient #6]), the CD4-positive, HIV-1 proviral DNA-positive T cells formed two subpopulations: one subpopulation with slightly decreased Leu 3A cell surface staining and the other with a phenotype approaching CD4 negative. In this group of patients, the decreased Leu 3A cell surface staining is statistically significant by χ^2 analysis ($P < 0.001$). The resolution into the two subpopulations following PCR-driven in situ hybridization and flow cytometry with Leu 3A approximated the dot plot distribution observed by flow cytometry with Leu 3A without target DNA amplification (data not shown). To screen for the range of values for mean peak fluorescence intensity, we evaluated the seven study subjects uninfected with HIV-1. Each determination was performed in duplicate. Among these seven subjects, the mean peak fluorescence intensity values ranged from 2.3 to 11.2. Because this represents a relatively wide variation in Leu 3A staining intensity for these uninfected patients, ascribing a virus-mediated modulatory effect to this specific parameter is potentially confounding.

To determine if the altered CD4 cell surface staining properties for cells harboring HIV-1 proviral DNA are a result of CD4 antigen downregulation or modification, split lymphocyte samples from four patients (patients 4, 5, 12, and 13) and from seronegative control patient 14 were stained with either anti-CD5, Leu 3A, or L120 antibody and analyzed for the presence

TABLE 2. Comparison of L120 mean peak fluorescence in the CD4-positive, HIV-1 DNA-positive and CD4-positive, HIV-1 DNA-negative subpopulations

Patient no.[a]	Mean peak fluorescence (L120-PE) of $CD4^+$ T cells which are[b]:	
	HIV^-	HIV^+
4	6.7 ± 0.3	7.5 ± 0.2
6	12.4 ± 0.4	12.7 ± 0.1
7	16.3 ± 0.1	17.2 ± 0.4
13	5.9 ± 0.2	5.3 ± 0.3
14	10.7 ± 0.3	0

[a] Patient numbers correspond to those in Table 1.
[b] Fluorescence is expressed in fluorescence units.

or the absence of HIV-1 product DNA. As noted above, there was concordance among the $CD4^+$ T-cell counts as determined by the total numbers, using anti-CD5 antibody, relative to the sum of the $CD4^+$ T-cell subpopulations, using Leu 3A or L120. In contrast to the decreased mean peak fluorescence observed for the CD4-positive, HIV-1 proviral DNA-positive T cells with Leu 3A, there was no statistical difference ($P = 0.14$) in the mean peak fluorescence observed for this population with L120 (Table 2 and Fig. 3).

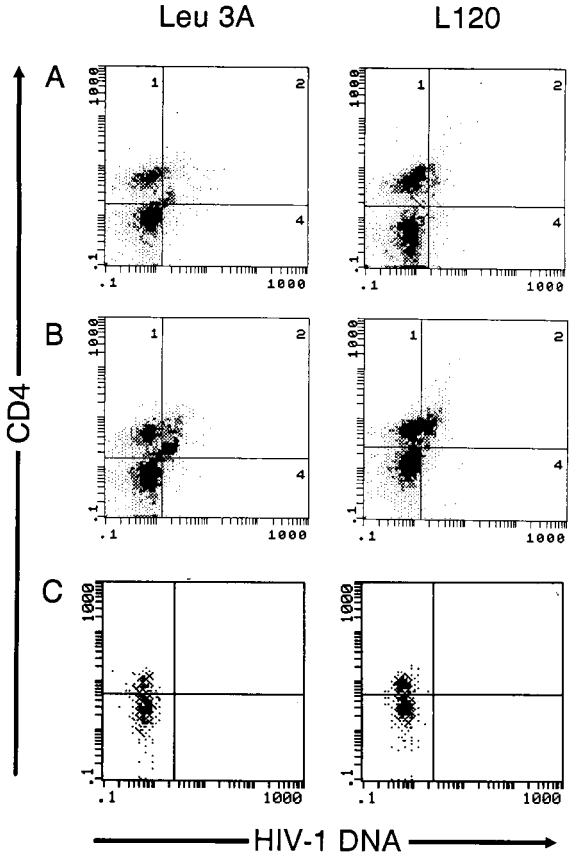

FIG. 3. Dot plots from samples from patient 13 (A) and patient 4 (B) showing a decrease in Leu 3A staining without a decrease in L120 staining in the CD4-positive, HIV-1 DNA-positive cell population when compared with the CD4-positive, HIV-1 DNA-negative cell population. The dot plots from a sample of an HIV-1-seronegative control patient (patient 14) (C) revealed no difference in intensity between Leu 3A staining and L120 staining. Ordinate, log red fluorescence. Abscissa, log green fluorescence.

DISCUSSION

HIV-1 infection of T cells and of cells of the monocyte/macrophage lineage requires gp120-mediated binding of the virion to the host cell CD4 surface molecule (28, 29). The CD4-gp120 interaction involves high-affinity binding of discontinuous sites of gp120 with the first immunoglobulin-like domain of CD4 (16, 26, 31, 32, 40). Changes in the molecular conformation of CD4 are presumed to allow direct fusion of the virus and host cell membranes that is mediated by the amino-terminal fusion domain of the envelope transmembrane glycoprotein (gp41) (32). The nucleocapsid core then enters into the host cell cytoplasm, where the virion is uncoated and the viral RNA molecule is reverse transcribed into DNA (16). After translocation to the cell nucleus, the viral genome can persist in a latent (44, 49), chronically infected (8, 9, 36), or productive state. A large proportion of these viral genomes are excluded from the replicating virus pool by virtue of being either genotypically or phenotypically defective (37).

The molecular events responsible for $CD4^+$ T-cell depletion and qualitative abnormalities of T-cell function have not been clearly delineated. Both indirect and direct immunopathogenic mechanisms postulated to explain these findings have been predicated upon the estimated numbers of HIV-1-infected cells (35). While a large proportion of infected cells would be compatible with direct HIV-1-induced cytopathicity, small numbers of HIV-1-infected cells require an indirect mechanism to account for the specific loss of the $CD4^+$ T-cell population. Therefore, determining the magnitude of the reservoir of virus in vivo and characterizing the virus-host cell interaction have significant implications for our understanding of viral pathogenesis.

In this study, we found that a significant proportion (up to 50%) of $CD4^+$ T cells can harbor virus in an HIV-1-infected individual. These results are comparable with those of earlier studies which also found that a large proportion of blood and tissue cells harbor proviral DNA (8, 34). One significant technical limitation to earlier reports was, however, that heterogenous cell populations were not fractionated to elucidate the numbers of infected cells which express the CD4 surface antigen. Quantification of proviral DNA in purified $CD4^+$ T-cell populations provided a wide range of estimates (1 in 10,000 to 1 in 10) of the proportion of blood cells that were infected with HIV-1 (20, 43). Cell homogenization and proviral DNA quantification predicted upon analysis of product DNA in solution have significant technical limitations compared with specific intracellular localization of viral sequences.

Our results confirm and extend the work of Bagasra et al. (1), who also found that a significant proportion of $CD4^+$ T-cells harbor proviral DNA. Although an alternative strategy was used in that study, the numbers of $CD4^+$ T cells containing proviral DNA are comparable in the two studies. Consistent with our earlier observations (36), we found evidence of HIV-1 proviral DNA in 3.6 to 15.6% of PBMCs. For the monocyte-depleted $CD4^+$ T cells, however, we found evidence of HIV-1 proviral DNA in 17.3 to 55.5% of cells. The difference in the proportion of infected $CD4^+$ T cells was not related to the subjects' $CD4^+$ T-cell counts. The apparent disparity between the stage of disease and the proportion of HIV-1-infected cells can be attributed to the fact that enumeration of the numbers of infected cells is a static measure of a dynamic process. Therefore, the relative proportion of cells containing virus that persists as a latent, chronically infected, or productively infected form and the dynamic equilibrium between these potential alternative states of infection were not assessed by these experiments. Understanding the transcriptional dynamics and kinetics of viral replication will require sequential measurements of viral gene expression in $CD4^+$ T cells before and after perturbation of viral replication with an efficacious antiretroviral agent. Additionally, since the vast majority of $CD4^+$ T cells are distributed in a larger tissue compartment throughout the body, there is a potential sampling error inherent in analyzing only blood cells, without accounting for virus residing in lymphoreticular tissue.

Simultaneous immunophenotyping and intracellular amplification also revealed a disparity in the cell surface staining characteristics observed for HIV-1-infected and uninfected cells in a single sample and for different CD4 epitopes in a split sample. To determine if HIV-1 affects CD4 cell surface expression or conformation in vivo, we performed CD4 epitope mapping with anti-Leu 3A antibody, which binds to an epitope overlapping the HIV-1 binding site in domain 1; anti-L120 antibody, which binds to a CD4 epitope in domain 4 that is unaffected by HIV-1 binding (17); and anti-CD5 antibody, a pan-T-cell marker also unaffected by HIV-1 infection. In the same patient sample preparation, simultaneous determinations of cell surface staining for CD4 by Leu 3A or L120 and intracellular HIV-1 product DNA revealed a 1.5- to 3-fold decrease in Leu 3A (CD4) staining in infected cells compared with that in uninfected cells. This observation is consistent with that of a previous study, which showed a decrease in anti-Leu 3A bead density (1). There was, however, no difference in the staining intensity of the L120 antibody for the two cell populations. This observation is consistent with those of previous studies which showed changes in CD4 and CD3 cell surface expression and associated functional alterations of the TCR in HIV-1-infected established cell lines (16, 25, 56). In one study, diminished Leu 3A cell surface staining in transcriptionally quiescent T cells coincided with their functional impairment, as determined by a sustained increase in cytosolic free calcium and activation of calcium-dependent endonuclease activity (25). Functional loss of the TCR-CD3 receptor has been found both in HIV-1-infected lymphocytes and in human T-cell leukemia virus-infected cells in vitro and in vivo (25, 55, 56).

While HIV-1-associated changes in CD4 cell surface expression may be a consequence of blocked antibody access to or conformational changes in the CD4 molecule, decreased CD4 transcription or translation, or intracellular CD4 sequestration, our results support the hypothesis that HIV-1 induces cell surface CD4 epitope masking. The epitope masking may be the result of gp120/gp160-CD4 interactions. This would account for the greater signals obtained with L120 than with Leu 3A; the former epitope is not occluded by gp120, while the latter epitope is occluded. Only two of these 13 HIV-positive patients (patients 5 and 6) had cells which expressed gp120-CD4 complexes as measured by CG-10 cell surface staining. Of these two patients, CG-10 cell surface staining was observed in approximately 9.1% of PBMCs from patient 5 and in 4.0% of PBMCs from patient 6 (Fig. 4B and C). Alteration of the cell surface staining characteristics may result from gp160 binding to CD4 before CD4 is expressed on the cell surface. The intracellular gp160-CD4 complex may, however, be recirculated to the endoplasmic reticulum without presenting to the cell surface. Previous studies have shown that intracellular CD4-gp160-$p56^{lck}$ complexes are OKT4 positive and OKT4A negative (OKT4A is an antibody that binds to an epitope similar to Leu 3A) (21). This phenotype is similar to the CD4 cell surface staining characteristics we observed for all of the HIV-1 DNA-positive study participants.

The subpopulation of $CD4^+$ T cells which exhibits the largest decrease in Leu 3A cell surface staining may be the population with the highest level of ongoing HIV-1 envelope gly-

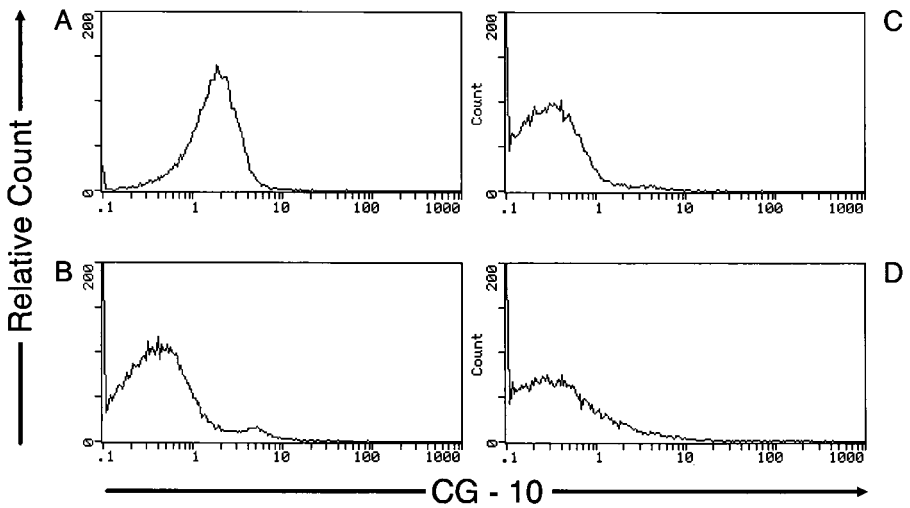

FIG. 4. Single parameter curves of PBMCs stained with CG-10, an antibody which binds CD4-gp120 complexes. HIV-1-infected CEM cells (A), a sample from patient 5 (B), and a sample from patient 6 (C) exhibit populations of cells which stain for CD4-gp120 on the cell surface. An HIV-1-seronegative patient sample (from patient 14) lacks a CG-10-positive cell population. Ordinate, relative cell count. Abscissa, log fluorescence.

coprotein expression, while the HIV-1-infected cells with little or no alteration in Leu 3A staining may harbor latent or genotypically or phenotypically defective forms with little or no gp120 expression. At the single-cell level, T-cell function may or may not be compromised, contingent upon the state of the viral genome. While productive infection will result in cell death, a transcriptionally active but nonproductive infection may produce T-cell dysfunction or anergy (19). Transient infection may not alter the CD4 molecule and, therefore, may not compromise T-cell function. The relative proportion of cells containing viral genomes in each of these alternative states may be a function of the dynamics of infection and the rate of disease progression. To test this hypothesis, multiplex PCR-driven in situ hybridization analyses will be required.

The significant proportion of $CD4^+$ T cells infected with HIV-1 and the associated alteration of the CD4 cell surface molecule in these infected cells are compatible with a direct role for the virus in HIV-1 pathogenesis. Alterations of the CD4 coreceptor as a consequence of gp120-CD4 binding can directly modify antigen receptor signal transduction (25). The interaction of the CD4 cytoplasmic domain with the myristylated tyrosine protein kinase $p56^{lck}$ is required for coreceptor-mediated signal transduction (14, 54). In the absence of coreceptor engagement, stimulation of the TCR results in a diminished response (53). Alternatively, interaction of the CD4 cytoplasmic domain with the myristylated viral protein Nef may result in direct CD4 downregulation or aberrant signal transduction (12). T-cell activation may, therefore, be inhibited as a consequence of the gp120-CD4 interaction. Perturbations of CD4 prior to TCR-mediated signal transduction may also induce apoptosis. HIV-1-mediated T-cell destruction by apoptosis can be induced in uninfected cells by exposure to soluble gp120 and then activation through the TCR (2). Additionally, apoptosis can be induced in T lymphoblasts acutely infected with HIV-1 (50). Although a soluble gp120-CD4 interaction is sufficient to induce apoptosis in uninfected cells in vitro, the large reservoir of persistently infected $CD4^+$ T cells with altered CD4 cell surface expression in vivo may account for programmed cell death following TCR activation. The functional significance of these potential pathways and their implications for HIV-1 immunopathogenesis remain to be determined.

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