LOSS OF CD4 MEMBRANE EXPRESSION AND CD4 mRNA DURING ACUTE HUMAN IMMUNODEFICIENCY VIRUS REPLICATION

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HIV is the only retrovirus that has as of now an identified receptor, the CD4 molecule (1–3), to which its outer membrane glycoprotein (gp110) binds with high affinity (4, 5). This glycoprotein is produced from the cleavage of a gp160 precursor generating gp110 and the gp41 transmembrane protein (6). This receptor/envelope interaction occurs in three distinct situations. Envelope gp110 expressed on a viral particle binds membrane expressed CD4 on target cells (4), controlling the host range and tissue specificity of virus infection (7–9); gp110 expressed on the surface of an infected cell, can bind to CD4 expressed on other(s) cell(s), inducing cell fusion and syncytium formation (10, 11). Finally, CD4 and gp110 can interact within a single cell, inducing the formation of immunoprecipitable gp110-CD4 complexes that are thought to account for the disappearance of CD4 from the cell surface of HIV-infected cells (7, 12). The presence of such receptor/envelope complexes in infected cells has been postulated for many other retroviruses, causing the so called "interference" phenomenon; i.e., cells infected with a given strain of avian leukemia virus (ALV)[1] are resistant to infection with other related but distinct strains of this virus (13). This is commonly viewed as resulting from the occupancy of the receptor, used by both retroviruses, by endogeneously produced envelope of the first strain. Since the nature of the receptor for these viruses is unknown, it has not yet been possible to verify this hypothesis. However, modification of the receptor synthesis at the transcriptional or post-transcriptional levels could also be involved in down-modulation of retrovirus receptor, as it has been proposed for HIV. Hoxie et al. (12) have shown that $CD4^+$ cells infected with HIV presented decreased levels of both CD4 mRNA and protein as compared with the initial uninfected cells after 2 to 3 mo of chronic viral replication. From these experiments, the authors could not discriminate between regulation of CD4 gene transcription or selection of low CD4-expressing subclones, more resistant to HIV cytopathic effect. In contrast, Stevenson et al. (14),

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[1] *Abbreviations used in this paper:* ALV, avian leukemia virus; HIV-VV, HIV vaccine recombinant virus; nt, nucleotide; PFU, plaque-forming units; RT, reverse transcriptase; SB, staining buffer.

using a subclone of the CEM line that was resistant to HIV cytopathic effect, proposed that upon HIV replication, a post-transcriptional mechanism affected CD4 protein production but also that of other T cell membrane markers. In both cases, these studies were conducted in established cell lines and did not necessarily reflect what really occurred in HIV-infected normal lymphocytes.

We have studied membrane expression and gene regulation of CD4 during acute HIV replication in normal CD4 lymphocytes as well as in CEM cells and after infection with HIV/vaccinia recombinant viruses (HIV-VV) expressing mutated *env* gene. We show that both CD4/gp110 complex formation and the rapid disappearence of CD4 mRNAs account for the modulation of the HIV receptor.

Materials and Methods

Preparation, Infection, and Culture of Cells. PBMC were obtained from HIV^- normal individuals, using the Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) separation method. When necessary, PBMC were depleted of $CD8^+$ T lymphocytes by specific cytotoxicity with mAb IOT8a (Immunotech, Marseille, France) and rabbit complement (Biokar, Pantin, France) before infection. After cytotoxicity, <1% of $CD8^+$ cells remain in the $CD4^+$-enriched PBMC. The CEM subclone (CEM 13) was isolated after cloning in semi-solid agar of the original CEM T-lymphoblastoid cell line.

Infection was performed with supernatant of cultured HIV-1-infected PBMC (LAV_{BRU} isolate (15), corresponding to the peak of viral production: $>5.10^5$ cpm/ml; $>10^5$ ID_{50} (16). PBMC or $CD4^+$-enriched PBMC were suspended for 1 h at 37°C (10^7 cells/0.1 ml HIV supernatant). Normal and infected lymphocytes were adjusted at 10^6 cells/ml and cultured at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1% antibiotics (penicillin, streptomycin, neomycin; Gibco-BRL, Uxbridge, UK) and 4 μg/ml PHA (PHA-M; Gibco-BRL). On day 3, cells were pelleted and resuspended in this complete medium containing IL-2 (20 U/ml) instead of PHA. Under these culture conditions, monocytes and B lymphocytes do not proliferate and at day 5, when we started analysis, <5% of non-T cells were present in the culture. CEM cells were cultured in the same medium without PHA and IL-2.

Viral production in the supernatant of HIV-infected cells was determined by particle-associated reverse transcriptase assay (17) and immunocapture ELISA (ELAVIA; Diagnostics Pasteur, Marnes-la-Coquette, France) using 10-fold dilutions of cell-free supernatants. Absorbance (A) was determined with an ELISA-plaque reader (Diagnostics Pasteur) at 492 nm.

Immunofluorescence Analysis. Control and infected cells ($2-5 \times 10^5$ cells per staining) were harvested, rinsed in cold PBS and incubated for 30 min at 4°C with appropriate mAbs diluted at 1:100 to 1:200 in staining buffer (SB) that comprised PBS, 0.5% BSA, and 0.05% sodium azide. mAbs used were as follows: anti-CD4: Leu3a PE (Becton Dickinson & Co., Mountain View, CA), T4-FITC (Coulter Corp., Hialeah, FL), OKT4 (Ortho Diagnostic Systems, Westwood, MA); anti-T cells: T3-RD1 (CD3), T11-RD1 (CD2), 4B4 RD1 (CDw29) (Coulter Corp.), Leu2a (CD8) (Becton Dickinson & Co.); anti-HIV: 41-1 (anti-gp41), 110-4 (anti-gp110) (Genetic Systems Corp., Seattle, WA). Unlabeled mAbs (OKT4, 41-1, 110-4) were revealed by sequential incubation with biotinylated sheep anti-mouse antibodies (Amersham Corp., Buckinghamshire, UK) at 1:25, and phycoerythrin-conjugated streptavidin (Becton Dickinson & Co.) at 1:25. When double-color labeling was performed (T4/OKT4, T4/41-1, T4/110-4), control ascites fluid (Dakopatts, Copenhagen, Denmark) at 1:25 was added to phycoerythrin-streptavidin in order to saturate the remaining binding sites of sheep anti-mouse antibodies before incubation with the second mAb, T4-FITC. After staining, cells were resuspended in SB containing 1% paraformaldehyde, kept at 4°C, and analyzed together with the FACS analyzer (Becton Dickinson & Co.). As described elsewhere (18), volume versus right-angle light scatter dot plot was used to gate viable cells and exclude debris as well as dead and giant autofluorescent cells. In initial experiments, the volume-scatter gate was correlated with propidium iodide staining and encloses a population with <5% of dead cells. Fluorescence

intensity was then determined within these gated viable cells and plotted in a 3-decade log-scale. Results are presented as histograms (cell number versus fluorescence intensity) for single-color staining experiments, or as autoscaled contour maps of cell number in double-color staining experiments, each axis representing fluorescence intensity in one channel (vertical, red; horizontal, green).

Cytoplasmic Extracts (Cytodots). Control and infected cells (2.5×10^5 viable cells as measured by trypan blue exclusion) were rinsed three times in cold PBS, resuspended in 100 μl of Tris (10 mM, pH 7.0), 1 mM EDTA, 2 mM Vanadyl ribonucleoside complex (Bethesda Research Laboratories, Gaithersburg, MD), 0.5% NP-40 (BDH Chemicals, Poole, England) and vortexed for 5 min on ice. Then, 10 μl of 5% NP-40 was added, cells were vortexed for 5 min on ice, and nuclei were pelleted by centrifugation at 4°C for 10 min at 10 000 *g*, 100 μl of supernatant was completed with 100 μl of 12× SSC (1× SSC NaCl 0.15 M, 0.015 M trisodium citrate, pH 7.0) 15% formaldehyde, incubated 15 min at 65°C and spotted on a nylon membrane (Hybond-N, Amersham Corp.) using the Minifold apparatus (Schleicher & Schuell, Dassel, Federal Republic of Germany).

Northern Blots. Total cellular RNA was extracted using the guanidinium thiocyanate/cesium chloride method (19). After centrifugation, RNA pellets were solubilized in 200 μl of 10 mM Tris, 1 mM EDTA, 0.1% SDS, pH 7.0, and were precipitated with 2.5 vol ethanol in the presence of 0,3 M ammonium acetate. Precipitates were washed with 70% ethanol, dried, and redissolved in 200 μl diethylpyrocarbonate-treated H_2O. RNA (10 μg per lane) was denatured for 15 min in 50% formamide, 20 mM Mops, 5 mM sodium acetate, 6% formaldehyde, pH 7.0, at 65°C, and was separated by electrophoresis in 1.1% agarose, 20 mM Mops, 5 mM sodium acetate gels as previously described (19). RNAs were transferred to nylon membranes (Hybond N, Amersham Corp.) using 20× SSC for 12–16 h.

Hybridization, Rehybridization Procedures. Nylon membranes (cytoplasmic extracts or Northern blots) were baked for 90 min at 80°C, prehybridized, and hybridized as described elsewhere (19). Specific probes of CD4 (pT4B) (3), HIV-1 (Sac I subclone of λJ19) (20), TCR-β_2 chain (4D1) (21) and β-actin (pAL41) (22) were α-^{32}P-radiolabeled using the random priming method (Multiprime DNA Labeling Systems; Amersham Corp.). Their specific activity ranged from 5 to 10 $\times$ 10^8 cpm/μg DNA. After hybridization, filters were washed under stringent conditions (three times in 2× SSC; 0.1% SDS at room temperature, and twice for 45 min in 0.1× SSC; 0.1% SDS at 65°C), and exposed to XAR-5 films (Eastman Kodak Co., Rochester, NY) in Quanta III cassette (DuPont de Nemours, Orsay, France) for 24–48 h. When necessary, filters were dehybridized for 2 h in 2 mM EDTA, 5 mM Tris, 1× Denhardt's (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA), pH 8.0, at 65°C, washed twice in 2× SSC, 0.1% SDS, and rehybridized as described above.

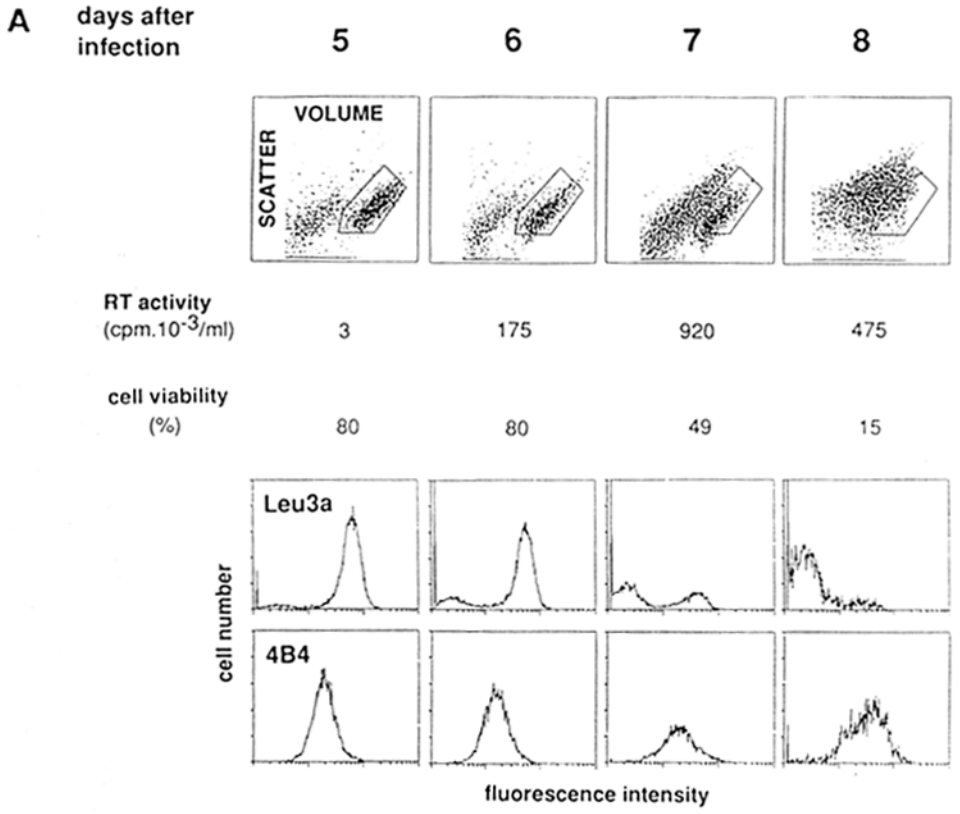
Construction of HIV-Vaccinia Recombinant Viruses. Manipulation of DNA was performed according to standard protocols and oligo-nucleotide-directed mutagenesis of subclones into M13 vectors was performed essentially as described (23). Construction of HIV-VV was realized as described (24). For HIV-VV 1135 the sequence encoding the signal peptide of the rabies virus glycoprotein gene (nucleotides [nt] 1–64 in Anilionis et al. [25]) was fused with the first codons of mature gp110 (nt 5857 in Wain-Hobson et al. [26]) using oligonucleotide-directed mutagenesis (amino acid sequence at the site of fusion: CFG.TNK) and the gp41 transmembrane domain (from nt 7834) was replaced by the rabies glycoprotein counterpart (nt 1387–1714; junction KIF.YVL). The sequence containing the two Lys-Arg cleavage sites between gp110 and gp41 has been altered from KAKRRVVQREKR to KAQNHVVQNEHQ (nt 7279–7314). HIV-VV 1138 is equivalent to 1135 except that the sequences corresponding to gp110 and the NH_2-terminal hydrophobic domain of gp41 have been removed by oligonucleotide-directed deletion (nt 5857–7398; junction CFG.QAF).

Infection with HIV-Vaccinia Recombinant Viruses. PHA-stimulated PBMC or $CD4^+$-enriched lymphocytes were infected with HIV-VV 1135, or 1138, or the wild-type vaccinia virus for 1 h at 37°C at a multiplicity of infection of 50 plaque-forming units (PFU)/cell. Cells were harvested at various times (see Fig. 3) and investigated for membrane proteins as described for HIV-infected cells. The efficiency of infection was monitored by immunofluorescence on fixed cells with antivaccinia mouse sera.

Results

Infection of stimulated $CD4^+$-enriched lymphocytes with HIV-containing supernatant induces virus replication that usually becomes detectable after a few days and peaks between day 6 and 8. The cell viability in the culture, as assessed by vital dye staining, begins to decrease within a day after initial viral detection. After the peak of viral replication, all cells ultimately die. To analyze the modification of cell surface marker expression during HIV replication, at a time when viral proteins are synthesized, it is therefore necessary to discriminate viable from dead cells. This can be routinely achieved by using a cell analyzer: viable cells can be distinguished from dead cells and debris on the basis of their volume and right-angle light scatter values (18). Using these two parameters, a gate can be drawn around the living cells cluster. Unlike dead cells, such gated cells exclude propidium iodide, a fluorescent vital dye, and are not stained by irrelevant antibodies. Thus, even at the time when massive death occurred in the infected cell culture, specific fluorescence could still be analyzed in the remaining viable gated cells, as shown in Fig. 1. In this experiment, till day 6 of the culture, most cells are viable. A day later, after the first detection of reverse transcriptase (RT) in the culture supernatant, the number of viable cells progressively decreased, as shown by both the scatter-volume dot plot and the count of cells excluding trypan blue. We have analyzed the expression of the CD4 molecule within the gated cell population using Leu3a mAb, and as a control we analyzed the expression of the CDw 29 molecule (27) detected by the mAb 4B4. We often used this mAb because CDw29 is also highly expressed on the $CD4^+$ CEM line for which we performed similar experiments. Throughout the culture time, no gross modification of CDw29 expression could be observed. On the contrary, gated cells expressing Leu3a gradually decreased in number and were almost absent at the end of the experiment. The rapidity of the appearance of these $Leu3a^-$,$4B4^+$ viable cells, as well as the absence of detection of $CD8^+$ cells (data not shown), rules out the possible emergence of a population of $CD4^-$ contaminating cells. Thus, loss of Leu3a staining could not be explained only by the death of HIV-replicating $CD4^+$ cells.

Co-expression of gp110 and CD4 on HIV Replicating $CD4^+$ Cells. To more precisely study modulation of CD4 at the surface of HIV-replicating cells we performed double-staining of $CD4^+$ enriched cells that were infected with HIV. Cells were labeled either with anti-gp41 or anti-gp110 mAbs or with different mAbs recognizing the CD4 molecule. Recently, amino acid substitution in the CD4 molecule allowed the localization of the epitopes recognized by the anti-CD4 mAbs as well as the HIV binding site. Leu3a (Becton Dickinson & Co.) and T4 (Coulter Immunology) recognize epitopes in the very vicinity or at the HIV binding site (28). They block HIV binding to CD4 and, reciprocally HIV blocks their binding to CD4 (4, 8, 29). OKT4 (Ortho Diagnostic Systems) recognizes an epitope more distant from HIV binding site and it does not interfere with the interaction between gp110 and CD4 (4, 8, 29). Using double-color staining procedure with OKT4, anti-gp41, or anti-gp110 mAbs on one hand (red, vertical axis) and T4 mAb on the other hand (green, horizontal axis), and after gating using volume-scatter dot blot as described in Fig. 1, we observed the emergence of three distinct populations of cells from the initially $T4^+OKT4^+gp110^-$ lymphocytes (Fig. 2): (*a*) $gp110^+OKT4^+T4^+$ cells; (*b*) $gp110^+OKT4^+T4^-$ cells; and (*c*) $gp110^+OKT4^-T4^-$ cells. Until day 4, infected cells



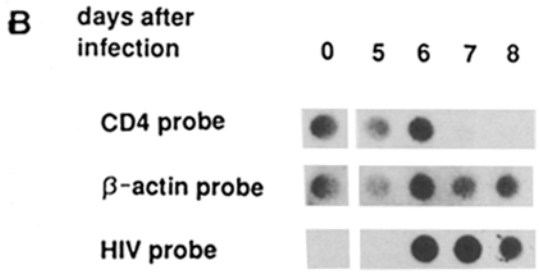


FIGURE 1. Cell death and CD4 expression during acute HIV replication in normal lymphocytes. PMBC were depleted of $CD8^+$ cells, infected with HIV, and cultured as described in Materials and Methods. At the indicated times, (*A*) RT activity in the supernatant, percentage of viable cells (trypan blue exclusion), cell surface labeling with Leu3a and 4B4 mAbs and (*B*) cytoplasmic extracts were performed. (*A*) Using a FACS, viable cells were first gated on the basis of their volume and scatter values (polygonal cluster in the dot-plot representation) as described. All samples were analyzed at the same time. Fluorescence was analyzed within these gated cells and represented as histograms: fluorescence (3-decade logarithmic scale) versus cell number. Fluorescence means of negative controls are comprised in the first decade (not shown). (*B*) Cytoplasmic dots spotted on nylon membranes, corresponding to 2.5×10^5 viable cells, were sequentially hybridized with CD4, β-actin and HIV probes and autoradiographed for 48, 24, and 24 h, respectively.

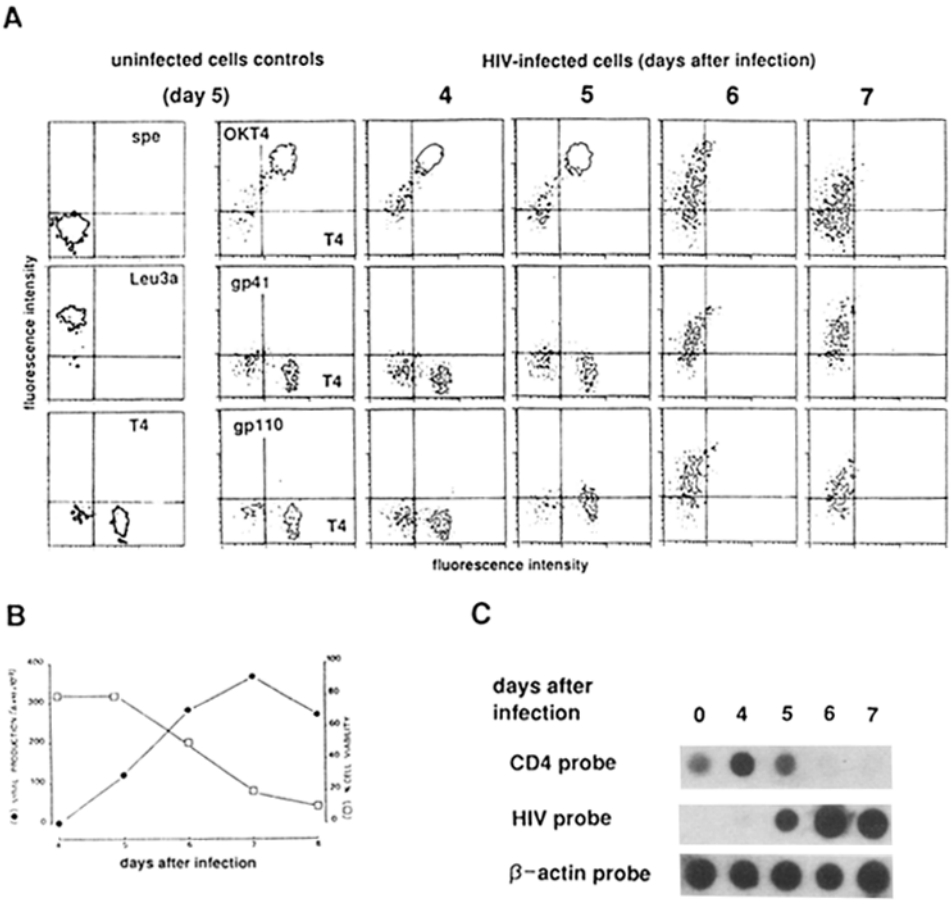


FIGURE 2. Coexpression of CD4 and HIV glycoproteins at the surface of HIV-infected lymphocytes. $CD4^+$ enriched lymphocytes were infected and cultured as described in Fig. 1. At the indicated times, (*A*) cell surface staining, (*B*) viral production in the supernatant (immunocapture ELISA), percentage of viable cells (trypan blue exclusion), and (*C*) cytoplasmic extracts were performed as described in Materials and Methods. (*A*) Infected cells (indicated time) and noninfected cells (day 5) were monitored for cell surface markers. Staining with OKT4 (CD4), 41-1 (gp41), and 110-4 (gp110) mAbs was analyzed in the red channel (phycoerythrin, vertical axis). T4 (CD4) was analyzed in the green channel (fluorescein, horizontal axis). Before fluorescence analysis, cells were gated on the basis of their volume and right-angle light scatter as described in Fig. 1 (not shown). Clusters represent auto-scaled contour graphs of cell number, and fluorescence intensity was plotted in 3-decade logarithmic scales. Quadrants were set using noninfected cells (day 5) stained with control antibodies (*left*): biotinylated sheep anti-mouse + phycoerythrin-streptavidin (spe) (red negative, green negative), Leu3a (red positive, green negative), and T4 (red negative, green positive). (*C*) Cytoplasmic dots on nylon membranes corresponding to 2.5 $\times$ 10^5 viable cells were hybridized with CD4, HIV and β-actin probes. CD4 and HIV probing were performed sequentially on the same filter. β-actin probing was performed on an independent filter. Autoradiography times were 48, 24, and 24 h, respectively.

kept their original $gp110^-OKT4^+T4^+$ phenotype. On day 5, a significant population of $gp110^+OKT4^+T4^+$ cells could be detected, few syncytia were noted and viral antigens detected by ELISA appeared in the supernatant. On day 6, at the time when significant loss of viability in the culture was observed, most cells were

gp110$^+$OKT4$^+$T4$^-$ while some were already gp110$^+$OKT4$^-$T4$^-$. At this time gp41 appeared at the cell surface. Subsequently, this latter population increased and cells finally died. In these experiments, the expression of control antigens CD2 and 4B4 remained unchanged at the cell surface of gated cells (data not shown). The almost complete disappearance of T4 staining in the face of the persistent OKT4 expression indicated that CD4 molecule was still present at the lymphocyte membrane at this time of viral replication (day 6), but in a configuration in which the epitope recognized by T4 mAb was no longer accessible. This, and co-expression of gp110, argued for the existence of CD4 in a complexed form with gp110. Indeed, gp110 binding to CD4 during adsorption of inactivated virions, or after incubation with purified gp110, induces similar modification of OKT4a but not of OKT4 staining (4, 30), and HIV gp/CD4 complexes can be immunoprecipitated by OKT4 but not OKT4a mAbs (12).

CD4 Expression after HIV-Vaccinia Recombinant Virus Infection. It was not possible from these experiments to determine to what extent gp110 was produced by infected cells and presented to their membrane before the assembly of viral particles, or was in the form of soluble gp110 passively adsorbed on uninfected CD4$^+$ cells, or perhaps at the surface of mature virions that were in the process of infecting new cells, both cases being responsible for CD4 masking (8, 30, 31).

To demonstrate that interaction between CD4 and HIV envelope actually occurred in each individual cell, we used vaccinia recombinant virus. Infection with such viruses allows the expression of foreign genes in vitro as well as in vivo (32) and is currently the best way to express high levels of normal or mutated HIV-*env* gene products in the infected cells (10, 11, 24, 33, 34). Initial experiments with normal *env* gene had shown an important shedding of gp110 from the cell surface of HIV-VV-infected cells (24). We therefore used a construction designed to optimize expression of HIV envelope at the cell membrane. In this construction (1135), the two cleavage sites that generate gp110 and gp41 from the gp160 precursor (Fig. 3 *A*) were inactivated by amino acid substitutions (see Materials and Methods). The signal peptide, the transmembrane and intracytoplasmic regions of HIV were replaced by those of rabies virus envelope glycoprotein. As control, we used a similar construction containing only the gp41 external region (1138) and also the wild-type vaccinia virus. High multiplicity of infection (50 PFU/cell) allowed synchronous, rapid, and massive infection of more than 90% of the cells, as determined by immunofluorescence with specific mouse sera to vaccinia antigens, further leading to cell death within 24–36 h. We first performed a time course experiment (Fig. 3 *B*) in which normal PBMC were infected by the two recombinants. Stainings using 4B4 and Leu3a mAbs were analyzed as described for HIV infection. While expression of a control molecule like CDw29 remained unchanged until 24 h after infection, a progressive decrease of Leu3a expression was observed after infection with HIV-VV 1135 but not with HIV-VV 1138. This effect, which appeared as early as 6 h after infection, could be clearly seen at 16 h and was almost complete at 24 h. Subsequent experiments were performed between 20 and 24 h after infection. We then analyzed expression of CD4 and HIV envelope after HIV-VV infection as we did after HIV infection (Fig. 3 *C*). Infection with the wild-type vaccinia or HIV-VV 1138 did not modify membrane expression of either Leu3a or OKT4 epitopes of the CD4 molecule, nor did they modify the expression of control molecules like CDw29 or CD3 (data not shown). Infection with

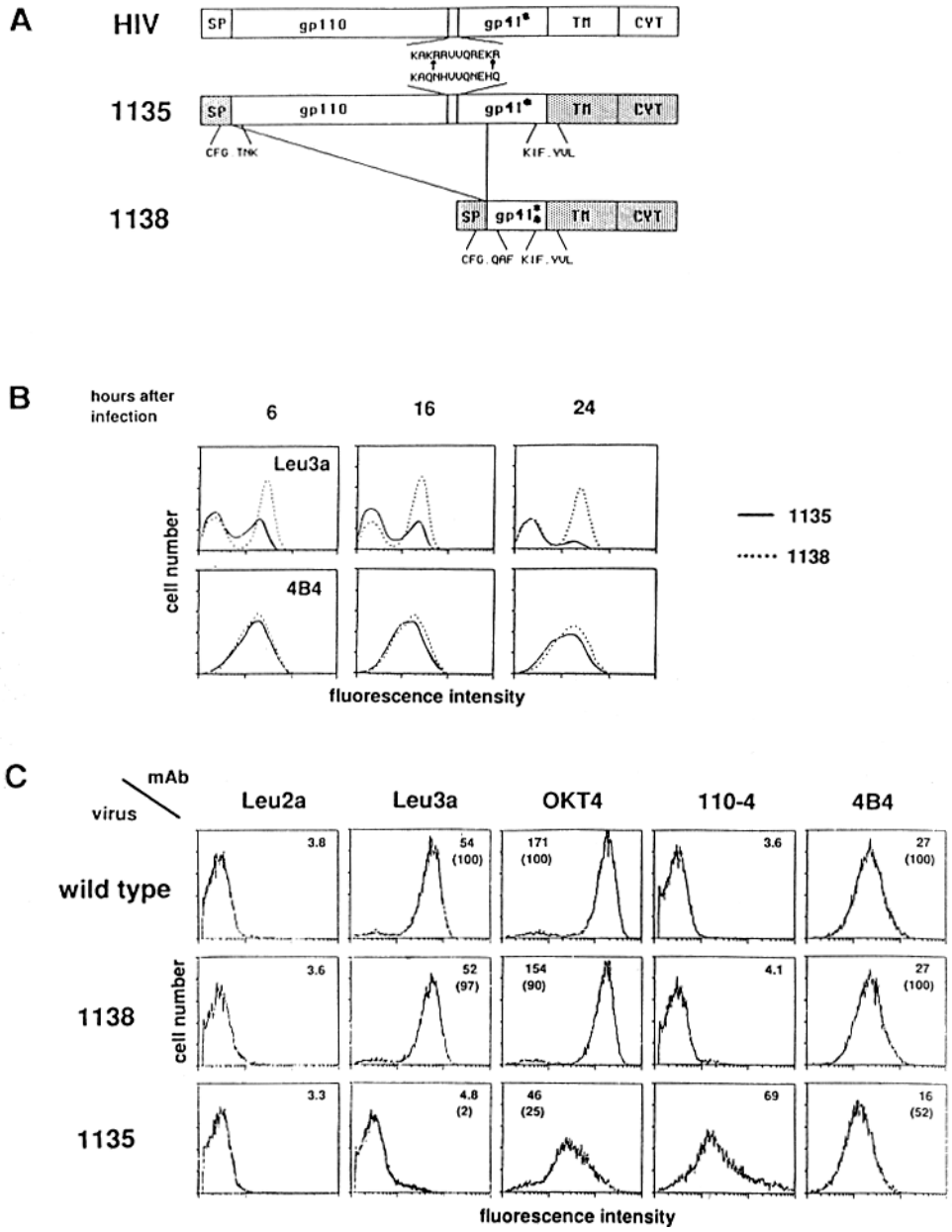


FIGURE 3. Expression of CD4 and HIV glycoproteins after infection with vaccinia recombinant viruses expressing HIV-env gene products. (*A*) HIV *env* gene (HIV) and sequences inserted in the thymidine kinase gene of HIV-VV 1135 (1135) and HIV-VV 1138 (1138) are represented. HIV sequences (*clear boxes*) and rabies virus sequences (*shaded boxes*) were fused as described in Materials and Methods. The whole gp41 sequence comprised boxes named gp41* (NH_2-terminal and external regions), TM (transmembrane region), and CYT (intracytoplasmic region). In HIV-VV 1138, gp41** represents the external region of gp41 fused with rabies glycoprotein signal peptide (SP, *left*) and transmembrane (TM) and cytoplasmic (CYT) rabies glycoprotein regions. Arrows indicate the two cleavage sites described in text and Materials and Methods. (*B*) At the indicated times, PBMC infected with HIV-VV 1135 or HIV-VV 1138 were labeled with Leu3a and 4B4

HIV-VV 1135 led to the expression of the hybrid envelope onto the cell surface that can be detected with anti-gp110 mAb. This was accompanied by the complete disappearance of the Leu3a epitope, while the reduction of OKT4 staining was about fourfold. In this experiment, a twofold decrease of 4B4 could be observed, a phenomenon that was not, however, consistently observed (Fig. 3 *B*). Since HIV glycoprotein produced by HIV-VV 1135, like its analogous construct lacking envelope cleavage sites, is not shed in the culture supernatant (35), it was concluded that in each individual cell the glycoprotein-CD4 interaction was mediated by endogeneously produced HIV glycoprotein.

CD4 Transcripts Are Lost during Acute HIV Replication. During HIV replication, the decrease and, finally, the complete disappearance of OKT4 staining strongly suggested the disappearance of existing CD4 molecules from the cell surface. CD4-gp110 complexes could be internalized or alternatively shed from the cell surface, but this also suggested a lack of new synthesis of CD4 molecules. One possible mechanism for the latter assumption could be the impairment of CD4 gene expression due to HIV replication. To investigate such a possibility, we analyzed CD4 mRNA during virus replication in T lymphocytes and CEM cells. In the two first experiments (Figs. 1 and 2) cytoplasmic extracts of 2.5×10^5 viable cells were prepared and spotted on nylon membranes at the times of immunofluorescence staining. Hybridization with a β-actin probe demonstrated that similar amounts of mRNAs were spotted. CD4 mRNA levels dramatically dropped on day 7 (Fig. 1) or on day 6 (Fig. 2), a day after HIV RNA and viral production could be detected. We next looked at mRNA levels by Northern blot analysis. Viral production and CD4 membrane expression were sequentially monitored in cultures of HIV-infected, or uninfected PBMC containing both $CD4^+$ and $CD8^+$ cells. Less than 4% of $CD4^-CD8^-$ cells could be detected throughout the culture period of control uninfected cells (Fig. 4). On the contrary, a significant proportion of $CD4^-CD8^-$ cells emerged in the infected culture; as demonstrated in the previous experiment, they arise from the originally $CD4^+CD8^-$ cells that had lost membrane CD4 upon viral replication. Total RNA was extracted each day from both infected and uninfected cells and 10 μg were analyzed by Northern blotting. Compared with β-actin, CD4 mRNA levels started to decrease in the infected culture on day 6, a time at which the decrease of $CD4^+$ cells and increase of $CD4^-CD8^-$ cells was observed. By day 7, when the proportion of $CD4^-CD8^-$ cells peaked, reaching approximately the percentage of cells that were originally $CD4^+$ and on day 8, CD4 mRNA levels became undetectable. Later, on day 9, CD4 mRNA remained absent, but at that time, all surviving cells were of the $CD8^+$ phenotype. We next investigated whether this dramatic disappearance of CD4 mRNA could also be observed in a $CD4^+$ T cell line, using a clone derived from CEM cells. This CD4 highly expressing clone (CEM 13) acutely replicates HIV and is highly sensitive to its cytopathic effects (Salmon, P., and D. Klatzmann, unpublished observations). As shown in Fig. 5, after HIV infection CD4

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mAbs and analyzed by FACS. (*C*) 20 h after infection with the indicated virus, $CD4^+$-enriched lymphocytes were labeled with Leu2a (negative control), Leu3a, OKT4, 110-4 (gp110), and 4B4 (positive control) mAbs, and were analyzed by FACS. For each sample, mean of fluorescence intensity and the relative fluorescence intensity were shown (calculated as follows): $100 \times [(mAb_{1135,1138} - Leu2a_{1135-1138})/(mAb_{\text{wild type}} - Leu2a_{\text{wild type}})]$.

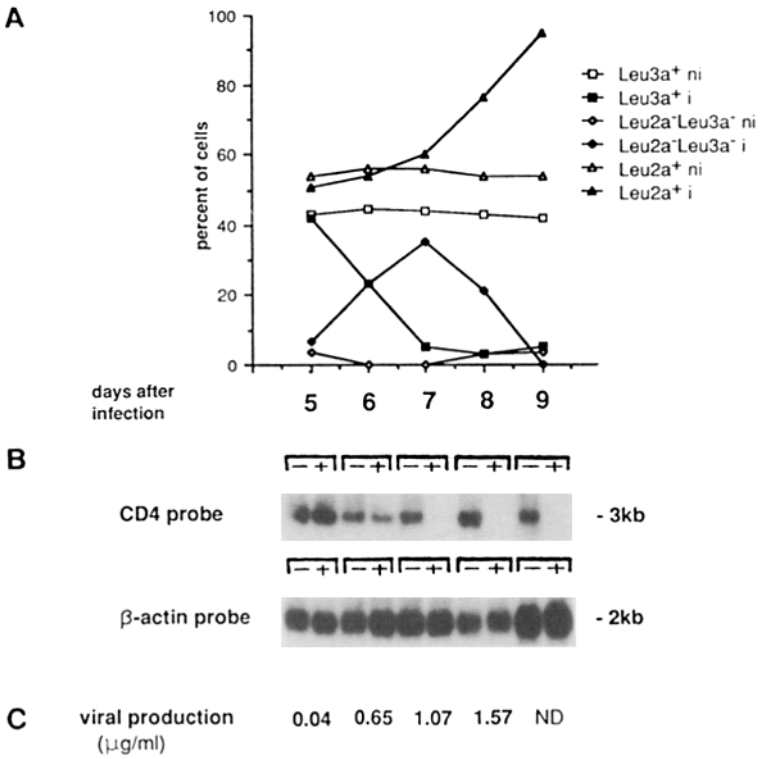

FIGURE 4. CD4 mRNA levels during acute HIV-replication in normal lymphocytes. At the indicated times, (*A*) cells were labeled with Leu2a (CD8) and Leu3a (CD4), (*B*) RNAs were extracted and analyzed by Northern blotting, and (*C*) viral production in the supernatant was determined using an immunocapture ELISA. (*A*) Percentages of noninfected (*ni*) and infected (*i*) cells expressing either Leu3a or Leu2a as well as $Leu2a^-Leu3a^-$ cells, are shown. $Leu2a^-$, $Leu3a^-$ cells arose from $Leu2a^-$,$Leu3a^+$ cells that have lost CD4 membrane molecules upon HIV replication. (*B*) Northern blot was performed as described in Materials and Methods, in both uninfected (−) and infected (+) cells. The nylon membrane was sequentially hybridized with CD4 and β-actin probes. Autoradiography times were 48 h.

transcripts were almost undetectable, when no more CD4 molecules were detectable on the cell surface as assessed by Leu3a and OKT4 staining. As controls, β-actin mRNA and a nonstructural protein mRNA coding for the TCR β chain were both present at slightly decreased or comparable amounts, respectively, in infected and uninfected cells. This indicated that CD4 transcript accumulation was specifically impaired during acute HIV replication in this clone of T-lymphoblastoid CEM line as for normal lymphocytes.

Discussion

When we initially reported the selective tropism of HIV for $CD4^+$ T lymphocytes (2), we already mentioned the striking disappearance of CD4 staining on the cell surface of infected cells. This observation has been reproduced and expanded and it is now well accepted that normal $CD4^+$ T lymphocytes, or T-lymphoblastoid cell lines, and $CD4^+$ monocyte/macrophages or monocytic cell lines, all lose CD4

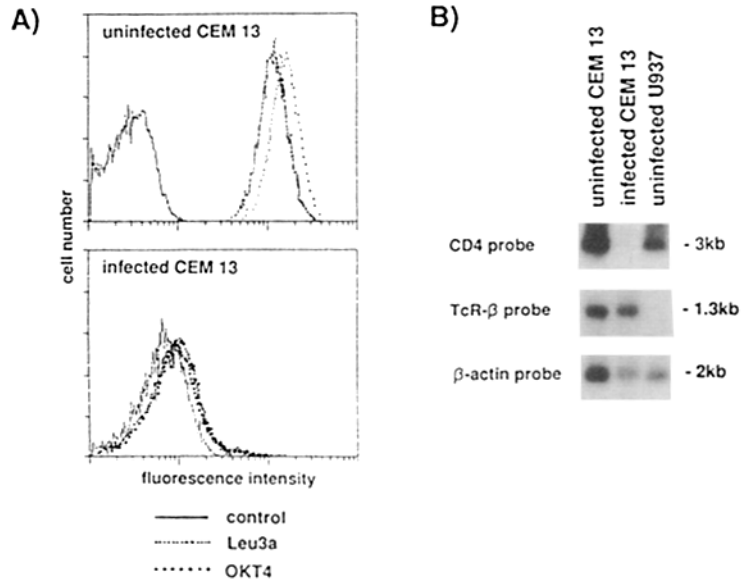

FIGURE 5. CD4 mRNA levels during acute HIV replication in a CEM clone. CEM-13 (CEM line-derived clone, see text) cells were infected and cultured as described in Materials and Methods. (*A*) At day 9, when viral production peaks (data not shown), cells were labeled with Leu3a and OKT4 mAbs. Control lines represent fluorescence histograms of cells treated only with biotin-coupled sheep antimouse antibodies plus phycoerythrin labeled streptavidin. (*B*) Northern blot analysis of uninfected (day 0) and infected (day 9) cells. The nylon membrane was sequentially hybridized with CD4, TcR β_2 chain, and β-actin probes. RNA from uninfected U937 (a monocytic cell line) was used as negative control for TcR β2 chain signal and positive control for CD4 and β-actin signals.

expression upon HIV-1 replication (12, 36, 37), but also upon HIV-2 and SIV replication (37). However, the mechanism of such a phenomenon is unclear. This could result from the HIV's cytopathic effect, which would first affect cells highly expressing CD4. Indeed, different $CD4^+$ cell lines or even individual clones from a given $CD4^+$ cell line appear to display heterogeneous sensitivity to HIV cytopathic effect, which correlates with the extent of CD4 expression (38). Impairment of CD4 expression at the cell surface could also be due to its interaction with gp110. Such an interaction could involve endogeneously produced gp110 (12) or alternatively soluble gp110 that is known to be released in the culture supernatant (24, 39, 40), or even virion adsorption to the cell membrane. Impairment of transcriptional (12) and post-transcriptional (14) events in CD4 gene expression have also been proposed as possible mechanisms. To investigate the mechanism of CD4 loss during HIV infection, it was therefore necessary to simultaneously monitor cell death, membrane expression of both CD4 and gp *env*, as well as CD4 mRNA amounts.

Although cell death rapidly occurred upon HIV replication in normal $CD4^+$ lymphocytes, this could not account for the acute loss of CD4 by this cell population. While it may be argued that these cells are already "sick" because of virus replication, we were able to identify a state in which these lymphocytes continued to express normal amounts of some membrane molecules such as 4B4 (which was chosen

because it is also highly expressed by CEM cells), or CD3 and CD2 (data not shown), while they had almost completely lost CD4 expression, as detected by Leu3a. The observation of CD4 down-modulation at a time when the cell can exclude a vital dye does not imply, however, that cell death is independent of CD4 impaired expression, which actually precedes cell death. Decreased expression of CD4 after chronic infection of established $CD4^+$ cells could well be due to the selection, among this population, of cells for which the equilibrium between HIV replication and cytopathic effect allows the emergence of a low CD4-expressing cell population (12). Stevenson et al. (14), using a subclone of the CEM cell line resistant to HIV cytopathic effect and with low CD4 expression, noted that CD4 loss was accompanied by decreased membrane expression of T3 (CD3), T11 (CD2), T8 (CD8), and T10 (CD38). This could indicate a different behavior of HIV in $CD4^+$ normal lymphocytes and cell lines and prompted us to use normal lymphocytes for subsequent experiments.

HIV gp *env* detection at the cell surface of viable infected cells has long been unsuccessful, and usually only immunofluorescence of fixed cells using HIV^+ human serum has been shown. The only cell surface staining with mAbs directed to gp110 has been reported by Lyerly et al. (30). However, in this report, gp110 staining was weaker on HIV-infected cells than on normal $CD4^+$ cells coated with gp110. We have developed a sensitive assay using well-characterized mAbs (41-1; 110-4) (41) and biotin-streptavidin amplification. This allowed us to observe that gp110 expression at the cell membrane precedes its interaction with CD4. The subsequent loss of T4 epitopes coexisting with the persistence of OKT4 epitopes is in line with results obtained by others in which binding of HIV or gp110, respectively, to the target cells induced the disappearance of Leu3a but not that of OKT4 epitopes (4, 30). This argues for the presence of CD4/gp complexes at the cell membrane. Hoxie et al. (12) have shown that such complexes can indeed be immunoprecipitated from HIV-infected cell extracts. They proposed that these complexes were intracytoplasmic because they could not detect membrane CD4 at the time of immunoprecipitation. Although we also have been able to immunoprecipitate such complexes at a time when we could not detect any membrane CD4 molecules (data not shown), our observation of the emergence of a $T4^-OKT4^+gp110^+$ cell population indicates that these complexes are actually present at the cell membrane at an early stage of HIV replication. However, it is impossible from these experiments to conclude if they have been formed at the membrane or in the cytoplasm or both. The observation that gp110 could first be detected before any change of Leu3a staining could suggest that they are in part formed at the membrane.

gp110 and gp41 are not associated by disulfide bonds (4) and it is known that soluble gp110 is released in the culture supernatant of infected cells (24, 39, 40). This and the production of viral particles could be the cause of the CD4/gp110 complex formation. To assess whether these complexes result from the interaction of endogeneously produced proteins, we used the advantage of infecting cells with HIV-VV, which allows high expression of normal or mutated *env* gene products at the cell surface. However, this system is limited by the rapid cell death induced by vaccinia replication. In the LAV_{BRU} isolate both Lys-Arg cleavage sites that can apparently be used to produce gp110 and gp41 from the HIV *env* precursor gp160 (35) were inactivated in the construct we used, and actually almost no soluble *env* could be detected in cell culture supernatant after infection by HIV-VV 1135. The use of

heterogeneous signal peptide transmembrane and cytoplasmic regions did not affect the correct expression of the construct at the cell membrane (35), which could still be detected by our mAb against gp110, but also which could effectively bind CD4. Similarly, HIV-VV 1138 led to a strong expression of recombinant envelope molecules that could be detected by anti-gp41 mAb (data not shown). Using these experimental infections, we demonstrated that, in the absence of released soluble envelope or virions, endogeneously produced *env* gene product and CD4 can associate to form complexes that are detected at the cell membrane. Whether they are membrane or cytoplasmic formed cannot be discriminated. Wild-type vaccinia infection, as well as HIV-VV 1138, induces cell death at a similar rate as that of HIV-VV 1135. The absence of any modification of CD4 expression with these two control infections provides additional evidence that cell "sickness," which may precede cell death as detected by vital dye staining, is not responsible for the observed phenomenon.

Our results contrast with those of Stevenson et al. (42). After transfection with an expression vector containing the *env* gene and a selection gene, the selected clones display 50% decrease of both OKT4 and OKT4a, compared with the original cells. However, while these authors could demonstrate cytoplasmic staining of acetone-fixed cells with a human serum, they did not demonstrate the presence of cell surface glycoprotein and thus, decreased OKT4 expression in their clones may be the result of selection.

In both HIV or HIV-VV infection, decrease of Leu3a by masking of this epitope by *env* gene products was followed by a decrease in OKT4 staining. This suggests the actual diminution of the number of CD4 molecules at the cell membrane, which can result from the protein interaction allowing either shedding or internalization of such complexes. However, after HIV-VV infection, OKT4 reduction is only partial, varying from 3-10-fold according to the experiments. This may be due to the rapid cell death induced by vaccinia or it can suggest that additional mechanisms are responsible for CD4 modulation during HIV replication. For instance, the sole expression of F (3'orf) gene product can cause the decrease of 30-50% of OKT4 staining. Because F protein presents homologies with oncogenic proteins known to activate protein kinase C, it has been proposed that F protein can induce CD4 internalization by its phosphorylation (43). In addition, Hoxie et al. (12) have observed a decrease of CD4 mRNA levels in cells from $CD4^+$ cell lines that survive chronic HIV replication. In contrast, Stevenson et al. (14) did not observe any important decrease in CD4 mRNA levels after HIV replication in a CEM clone that was resistant to HIV cytopathic effect. In both cases, observations were made on cells resistant to HIV cytopathic effect, and thus behaved differently from normal $CD4^+$ lymphocytes. For these reasons, we looked to CD4 mRNA during acute HIV replication in normal $CD4^+$ lymphocytes and in a CEM clone. We always observed a dramatic decrease in the level of these transcripts in both cases. The kinetics of the observed disappearance of CD4 mRNAs usually showed that the first significant decrease occurred on the day when HIV became detectable either by RNA detection, RT or antigen measurement, and surface expression of HIV gp110. The day after, this disappearance was almost complete, while at least 50% of the initial cultured cells were still viable. This very rapid decrease makes it unlikely that a selective process occurs, in which cells containing very high CD4 mRNA levels would first disappear, leaving a cell population expressing extremely low or undetectable levels of these mRNAs.

In these experiments, the number of viable cells from which we prepared extracts in the blot experiments was calculated after trypan blue exclusion. Hybridization with a β-actin probe also provided an internal control for the amount of mRNA spotted or electrophoresed. In addition, the 1.3-kb TCR β chain mRNA, which is produced after a complex transcriptional and post-transcriptional regulation (44, 45), was normally synthesized in these cells. The mechanism of disappearance of CD4 mRNA has yet to be elucidated. Events preceding cell death could lead to a more rapid degradation of CD4 mRNA than of other mRNAs. Alternatively, viral proteins could directly or indirectly regulate CD4 mRNA synthesis. It has already been demonstrated that NFκB, a cellular factor involved in the regulation of immunoglobulin synthesis, can activate HIV enhancers (46). It would be an interesting possibility that, on the other hand, viral proteins could regulate the CD4 gene, a member of the immunoglobulin supergene family. Experiments are currently in progress to investigate these alternative possibilities.

In any case, at the time viral particles are produced, CD4 molecules are absent from the cell surface. The question then arose: Why did HIV regulate the level of its receptor expression both at cell membrane and mRNA levels? This phenomenon is reminiscent of the well-described viral interference, in which cells that are chronically infected by a virus are protected from reinfection by another distinct strain that uses the same cellular receptor (13). Similarly, in the myxovirus family, neuraminidase of influenza virus, previously known as "receptor destroying protein," removes sialic acid from the membrane of the host cell (47) that is used as receptor by the virus (48). Mutants of influenza virus that possess a non-functional neuraminidase are less infectious probably because of their reuptake by the remaining receptors (49). In the case of HIV, virus envelope and receptor are coexpressed by the infected cell and their cytoplasmic and/or membrane interaction will not allow the expression of a sufficient amount of free *env* gene products, possibly decreasing the production and infectivity of virus particles. A selective advantage could thus have led HIV, and perhaps other retroviruses, to develop this multiple mechanism to downregulate its receptor during replication.

Given the nature of HIV receptor, if mRNA loss corresponds to an actual regulation of gene expression, such a complete shut-off of CD4 expression suggests the interesting possibility that HIV could use mechanisms similar to those used in normal cellular T cell differentiation during which CD4 and CD8 genes may be sequentially turned on and off (50). Our observations may thus provide new insights into these mechanisms of regulation.

Summary

Using mAbs and genomic probe to the CD4 molecule, the HIV receptor, we demonstrated that HIV replication induces the disappearance of its functional receptor from the cell surface by two distinct mechanisms. First, after being expressed onto the cell surface, HIV envelope gp110 will complex CD4, efficiently masking the CD4 epitope used by the virus to bind its receptor. This phenomenon occurs on the surface of each infected cell and is not due to the release of soluble gp110; infection with recombinant HIV/vaccinia viruses expressing a mutated HIV *env* gene designed to prevent gp110 release from the cell surface induces a similar gp/CD4 complexes formation. Second, virus replication induces a dramatic and rapid loss of CD4 mRNA

transcripts, preventing new CD4 molecules from being synthesized. These two mechanisms of receptor modulation could have been developed to avoid reinfection of cells replicating the virus as well as to produce more infectious particles. These results suggest that the classical virus interference documented for other retroviruses might not only be due to receptor/envelope interaction, but might also depend on receptor gene expression.

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