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An antibody that binds the immunoglobulin CDR3-like region of the CD4 molecule inhibits provirus transcription in HIV-infected T cells

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We used the polymerase chain reaction (PCR) to study which step(s) of the human immunodeficiency virus type 1 (HIV-1) life cycle may be blocked following treatment of HIV-exposed CEM cells with 13B8-2, a monoclonal antibody (mAb) specific for the immunoglobulin (Ig) CDR3-like region of the CD4 molecule and able to inhibit the productive infection of CEM cells by HIV-1. The presence of viral RNA was investigated and found in 13B8-2 mAb-treated CEM cells 30 min after viral exposure; the full-length viral DNA was found at 24 h post-infection. We also found integrated forms of viral DNA at 24 h post-infection. However, the integrated provirus was transcriptionally inactive in 13B8-2 mAb-treated cells, as demonstrated by the absence of spliced HIV-1 mRNA. The lack of HIV transcription under 13B8-2 mAb treatment was confirmed by chloramphenicol acetyltransferase (CAT) assay. We conclude that the inhibition of viral gene transcription accounts for the lack of progeny virions in culture supernatants of cells treated with this anti-CD4 mAb. We also demonstrate that 13B8-2 blocks viral production from chronically infected cells and restores CD4 cell-surface expression on CEM cells containing an integrated provirus(es). We found this effect to be reversible. Moreover, we demonstrate that 13B8-2 mAb treatment is efficient on different HIV-1 and HIV-2 virus isolates. These results may have major implications for the treatment of AIDS.

Key words: CD4/HIV/transcription

Introduction

The human immunodeficiency virus type 1 (HIV-1) is the primary etiological agent of acquired immunodeficiency syndrome (AIDS) and associated diseases (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984). In individuals infected with HIV, the $CD4^+$ T lymphocyte is one of the major cell types infected by the virus (Klatzmann *et al.*, 1984), and progression towards AIDS is mainly characterized by the depletion of the $CD4^+$ T-lymphocyte population.

Infectious uptake of HIV into these cells is a complex process initiated by high-affinity binding of the virus envelope glycoprotein gp120 to the cellular receptor CD4 (Smith *et al.*, 1987; Fischer *et al.*, 1988). The molecular events that follow binding and underlie fusion remain poorly understood. Fusion may be mediated by specific CD4-induced changes in virion spike structure that activate the fusogenic property of the hydrophobic amino terminus of the envelope glycoprotein transmembrane subunit (gp41) (McCune *et al.*, 1988). This process appears to be pH independent (Stein *et al.*, 1987).

Once inside the cell, HIV reverse transcriptase (RT) catalyzes the synthesis of different forms of double-stranded proviral DNA from the single-stranded dimeric retroviral RNA genome (Peliska and Benkovic, 1992). Circular proviral DNA forms could simply be by-products of retroviral replication, whereas linear DNA molecules are likely to be the immediate precursors for retroviral integration (Brown *et al.*, 1987; Roth *et al.*, 1989). It is obvious that the activation state of the target T cell greatly influences viral replication. Although HIV can enter resting T cells (Zack *et al.*, 1990), mitogenic stimulation is required to ensure complete reverse transcription and/or integration and to induce subsequent progeny virus production (Tong-Starksen *et al.*, 1989; Stevenson *et al.*, 1990a,b; Zack *et al.*, 1992).

The integrated provirus is flanked by long-terminal repeats (LTRs) generated during the process of reverse transcription. The 5′ LTR functions to promote proviral transcription and contains recognition sequences for several constitutively expressed or inducible host cell transcription factors (reviewed by Gaynor, 1992), whereas the 3′ LTR is required for efficient polyadenylation of the transcripts. When transcription begins, the multiply spliced (2 kb) species appear first and encode the regulatory proteins Tat, Rev and Nef. Later on, the singly spliced (4.3 kb) species encode the envelope proteins, and the unspliced (9.2 kb) HIV-1 RNA species function as both the viral genomic RNA and as mRNA for the Gag-Pol and Gag proteins (Kim *et al.*, 1989; Pomerantz *et al.*, 1990). The mRNA is translated by free cytoplasmic ribosomes. The last steps of replication include assembly of new virions and release of particles: the capsid precursor protein and the capsid replicative enzyme precursor proteins co-assemble at the inner surface of the cell membrane; the capsid precursor binds to viral RNA by its packaging sequence and the complex assembles into a spherical particle. The virion matures during budding and release from the cell surface. Late stages of the HIV replicative cycle may be accompanied by the death of the host cell, a mechanism known as syncytia-independent single cell killing (Stevenson *et al.*, 1988). Another cytopathic effect of HIV has been mainly described *in vitro* and consists of syncytium formation following CD4/gp120 interaction and membrane fusion (Lifson *et al.*, 1986; Sodroski *et al.*, 1986).

There are an increasing number of reports showing that anti-CD4 monoclonal antibodies (mAbs) may interfere with the virus life cycle either by inhibiting virion-anchored gp120 binding to cell surface CD4 during the infection process, or by inhibiting syncytia formation, or by interfering with both events. Recently, several studies have suggested that

some anti-CD4 mAbs specific for either domain 1, domain 2 or domains 3/4 of CD4 do not block CD4/virion-anchored gp120 binding, but efficiently interfere with the HIV replicative life cycle at a post-binding step (Burkly *et al.*, 1992; Hasunuma *et al.*, 1992; Moore *et al.*, 1992b; Rieber *et al.*, 1992; Corbeau *et al.*, 1993). Moreover, Rieber *et al.* (1992) have shown that HIV infection of cells can be prevented *in vitro* by the CD4 mAb M-T413 even when added as late as 30–120 min of culture at 37°C after cellular contact with virus, suggesting this mAb inhibited a post-fusion step of the virus life cycle.

In this study, we further characterize the nature of 13B8-2 mAb-mediated inhibition of viral particle production and demonstrate for the first time that an anti-CD4 mAb may block provirus transcription.

Results

Experimental model for in vitro infection of CEM cells

We recently observed that 13B8-2, an anti-CD4 mAb reacting with the immunoglobulin (Ig) CDR3-like region of the CD4 molecule, inhibits HIV-1 particle production by CEM cells (Corbeau *et al.*, 1993) and peripheral blood mononuclear cells (P.Corbeau and M.Benkirane, unpublished observations), although it fails to block virion binding to target cells. To investigate the HIV-1 life cycle in CEM cells cultured in the presence of 13B8-2, CEM cells were exposed for 30 min at 4°C to 100 50% tissue culture infective dose ($TCID_{50}$) of HIV-1 stock solution previously treated with 100 U/ml of RNase-free DNase. They were then extensively washed and cultured at 37°C in the absence of mAb or in medium containing 10 μg/ml of 13B8-2 (see Figure 1). Control cultures consisted of cells exposed to heat-inactivated viral particles, cells treated with saturating concentrations of the anti-CD4 mAb OKT4A before being exposed to HIV-1 (because OKT4A inhibits viral particle binding to cell-surface CD4), cells treated with saturating concentrations of the anti-CD4 mAb OKT4 after viral exposure (because OKT4 does not inhibit particle binding and thus served as a negative control for interference with post-binding steps), and cells treated with 3′-azidothymidine (AZT) before and after viral exposure (because AZT interferes with reverse transcription of HIV). As shown in Figure 1, 13B8-2 completely blocked viral production during the first 14 days of culture, whereas viral production was apparent after 7 days in culture in the absence of mAb or in medium containing OKT4. Culture supernatants of cells exposed to heat-inactivated virus remained virus free after 14 days of culture. Viral particles were produced after 10 days of culture in supernatant of cells exposed to HIV-1 and treated with 10 μM AZT, suggesting that RT had bypassed the blockade of retrotranscription under the experimental conditions used. All molecular studies below were performed on cells harvested at different times from these cell cultures, meaning that results can be analyzed with respect to the RT activity data illustrated in Figure 1.

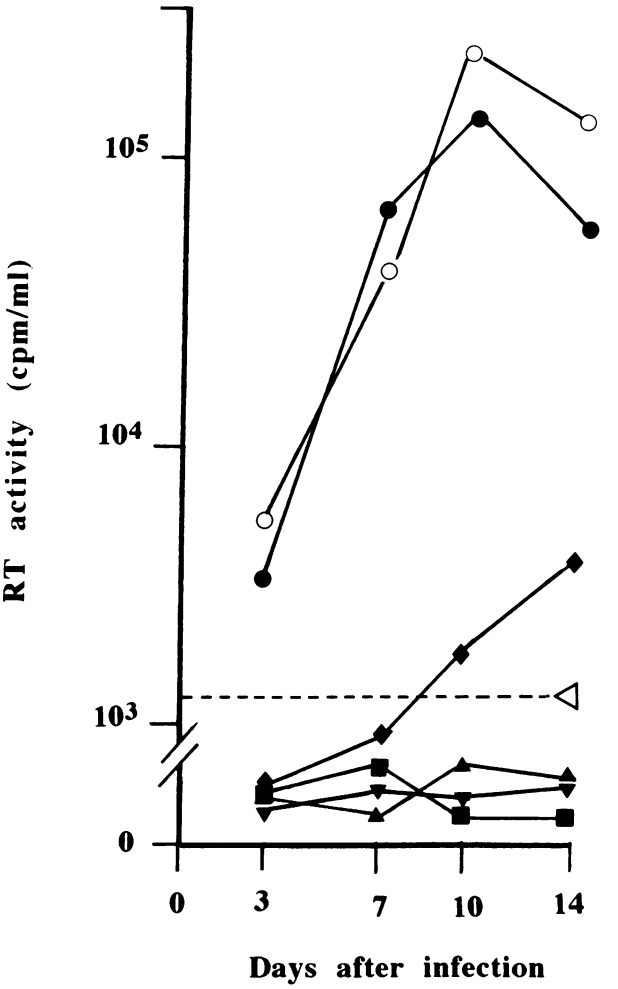

Fig. 1. Inhibition of viral particle production by anti-CD4 mAb added to the culture medium of HIV-1-exposed CD4+ cells. CEM cells were exposed to 100 $TCID_{50}$ of HIV-1Lai, washed and cultured in the presence of 10 μg/ml anti-CD4 mAb 13B8-2 (■) or OKT4 (●), 10 μM AZT (◆), or in medium alone (○). Viral production was followed by measuring RT activity in the cell-free culture supernatant. RT activity $< 1.5 \times 10^3$ c.p.m./ml was considered as negative (dashed line). RT activity measured in the supernatant of cells exposed to heat-inactivated HIV-1 (▲) or treated with OKT4A at 2 μg/ml before viral exposure (▼) is also shown and was used as a control for PCR assays.

Failure of 13B8-2 mAb to inhibit HIV-1 entry into T cells

To determine if the lack of virus production was due to a lack of virus entry into treated cells, the presence of viral RNA during the first 30 min following viral exposure was studied by RT-polymerase chain reaction (PCR). Complementary DNA (cDNA) was made using an oligo (dT) primer and AMV reverse transcriptase; cDNA products were subjected to PCR analysis. The primer pair M667/M668 was used to study the presence of viral RNA in cells exposed to HIV-1Lai and treated with anti-CD4 mAb. This pair will detect all HIV-1 RNAs (see Figure 2 for explanations and the approximate location of oligonucleotide primers). PCR amplification using the primer pair TK I/TK II was used as an internal control for RNA extraction. No viral RNA was detected in cells exposed to heat-inactivated virions (Figure 3, line 5), or cells treated with OKT4A before viral exposure (line 11). Moreover, as expected, no signal was found for samples which were not submitted to AMV-reverse transcription. Viral RNA was indeed detected in cells exposed to HIV-1 and cultured in the absence of mAb (line 9) or cultured in the presence of AZT (line 7), or in the presence of anti-CD4 mAb OKT4 (line 13). Similarly, HIV-1 RNA was detected in cells exposed to HIV-1 and cultured in the presence of 13B8-2 (line 15). This RNA was

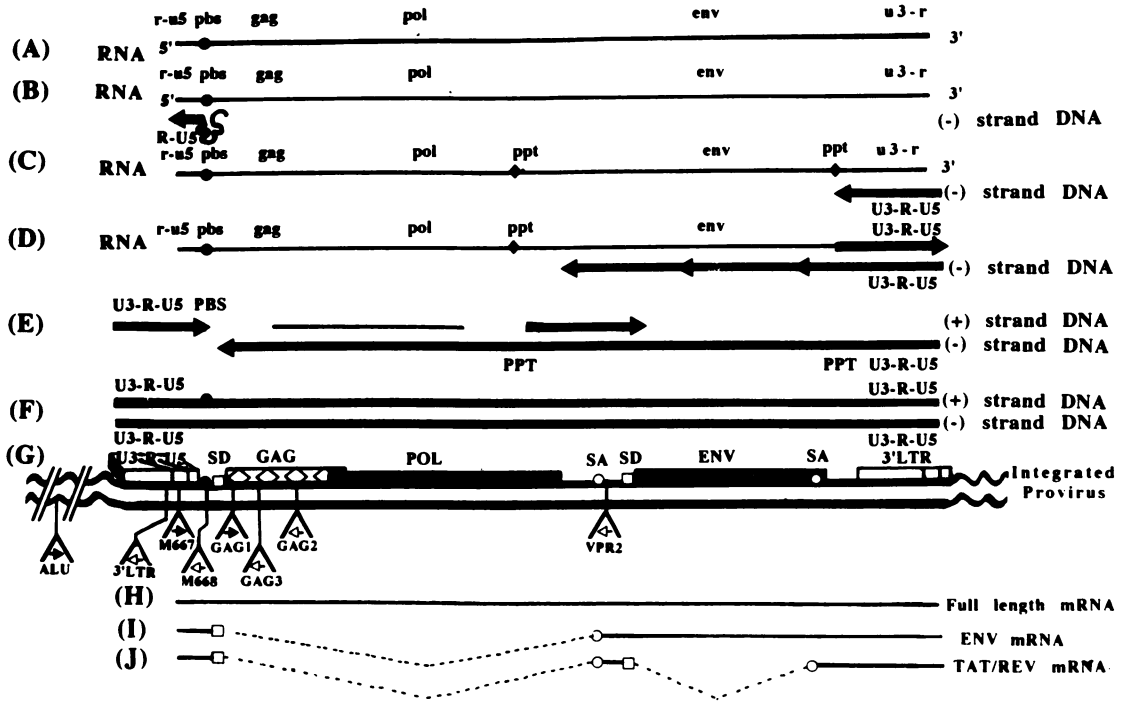

Fig. 2. Schematic diagram of reverse transcription, integration, and transcription and location of HIV-1-specific oligonucleotide primers for PCR analysis. Reverse transcription is schematized according to Hu and Temin (1990). Bold lines represent DNA and thin lines represent RNA. Parts of the LTRs, including the promoter 5′ (u3), are absent in viral RNA (**A**) and are regenerated during reverse transcription. A tRNA annealed to primer binding site (pbs; ●) of viral RNA is used as a primer for DNA synthesis (**B**) of minus (−) strand strong stop DNA. After (−) strand strong stop DNA has been transferred to the 3′ end of the RNA (**C**), (−) strand DNA synthesis continues while RNase H makes a nick at the polypurine tract (ppt; ◆). The (+) strand DNA synthesis begins (**D**) then the (+) strand strong stop DNA transfers to almost completed (−) strand DNA (**E**) and DNA synthesis is finished (**F**). Under appropriate conditions, the retroviral genome is integrated into the host cell genome (**G**) and finally mRNA synthesis occurs giving rise to different mRNA species (**H, I, J**). The splice donor (SD; ○) and splice acceptor (SA; □) regions are indicated. The location and orientation of HIV-1-specific oligonucleotide primers used for PCR analysis is aligned with the provirus (G). The primer orientations are indicated by arrows (closed symbols for the sense oligonucleotides and open symbols for the antisense oligonucleotides). HIV-1-specific oligonucleotide primers used in PCR to detect viral RNA are (i) the oligonucleotide primer pair M667/M668 previously described (Zack *et al.*, 1990) which detects both full-length and spliced HIV-1 mRNA molecules (Tat/Rev and Env) as a 161 bp fragment, and (ii) the oligonucleotide primer pair M667/VPR2 which will detect only spliced mRNA species (although both spliced and unspliced mRNA forms were fully retrotranscribed by the AMV-RT, and could serve as matrix for PCR, the distance of 5383 bp in between M667 and VPR2 on the product of retrotranscription of the unspliced mRNA was not compatible with efficient PCR reaction under our experimental conditions) and form 320 bp PCR fragments. HIV-1-specific oligonucleotide primers used in PCR to detect viral DNA are M667, M668, GAG1, GAG2 and GAG3. The oligonucleotide primer pair M667/M668 will detect all intermediate molecules formed during reverse transcription as 161 bp PCR fragments. The oligonucleotide primer pair M667/GAG3 will only detect the full-length DNA resulting from complete reverse transcription (because gag region is synthesized late during reverse transcription) as 674 bp PCR fragments. The oligonucleotide primer pair GAG1/GAG2 will detect a late stage of retrotranscription as 719 bp PCR fragments. The oligonucleotide *Alu*I (repeated sequence found in eukaryotic genomes) was used to test integrated viral DNA. The oligonucleotide primer pair *Alu*I/M668 form PCR-amplified products of various sizes with respect to the distances that separate the HIV-1 5′ LTR from its upstream Alu sequences.

probably due to virions that had bound to the cell surface or been internalized.

Failure of 13B8-2 mAb to inhibit HIV-1 reverse transcription

We next used direct PCR to investigate the reverse transcription process which is thought to proceed through a series of steps according to the accepted model of Hu and Temin (1990) (see Figure 2 for a schematic diagram of reverse transcription and approximate location of oligonucleotide primers). The presence and structure of HIV-1 DNA in infected cells was studied by mean of different oligonucleotide primer pairs, M667/M668 and M667/GAG3, respectively, which will detect either all intermediate molecules formed during reverse transcription or full-length viral DNA only. PCR amplification using the primer pair β-globin I/β-globin II was performed as an internal control for DNA extraction. We loaded various amounts of extracted DNA and HIV-1 DNA standards onto the gel to estimate the number of HIV copies present in the samples tested. Using the M667/M668 oligonucleotide primer pair, HIV-1 DNA was found at 6 h after viral exposure in cells cultured in the absence of mAb (Figure 4A, lines 4−6) or cultured in the presence of anti-CD4 mAb OKT4 (lines 7−9). No HIV DNA was found in the controls (lines 1−3). Use of heat-inactivated virus showed that contaminating viral DNA in the DNase I-treated virion preparation (see Materials and methods) was minimal (line 2). Under these experimental conditions, HIV-1 DNA was found in cells cultured in the presence of 13B8-2 (lines 10−12).

The results were similar at 24 h post-infection except for HIV-1 DNA copy number, which seemed to increase with time (Figure 4B), probably owing to an increasing number of viral RNA molecules reverse transcribed into cDNA and full-length DNA. At 24 h post-infection, a weak band was found in cells exposed to HIV-1 under AZT treatment (line 3); it probably corresponds to viral DNA synthesized from a limited amount of retrotranscribed HIV DNA.

The extent of reverse transcription in CEM cells was tested using the M667/GAG3 oligonucleotide primer pair that detects complete or nearly complete reverse-transcribed HIV DNA molecules. As shown in Figure 4C, 24 h after viral exposure we found very low amounts of full-length viral DNA in the sample prepared from cells exposed to HIV-1 under AZT treatment (line 3), and found evidence for complete synthesis of full-length DNA in cells exposed to HIV-1 and cultured in the absence of mAb (lines 4–6) or cultured in the presence of anti-CD4 mAb OKT4 (lines 7–9). Similarly, full-length HIV-1 DNA was found in cells exposed to HIV-1 and cultured in the presence of 13B8-2 (lines 10–12). These results demonstrate that 13B8-2 mAb treatment did not affect reverse transcription.

Failure of 13B8-2 mAb to inhibit integration

To investigate whether or not 13B8-2 mAb treatment affects the integration of the full-length viral DNA, high molecular weight DNA was extracted from cells and analyzed by PCR using the oligonucleotide primers *Alu*I/M668 (M668 oligonucleotide was 5′ end-labeled with ^{32}P). This primer pair will generate DNA fragments of various sizes depending upon the provirus integration site(s) and the distances that separate the 5′ HIV-1 LTR of the integrated provirus from the upstream Alu sequences of the eukaryotic genome. PCR amplification fragments were electrophoresed, transferred onto a nylon sheet and analyzed by autoradiography. As shown in Figure 5A, radioactive PCR fragments of various sizes were obtained in samples prepared from CEM cells exposed to HIV-1 and treated with OKT4 (line B), or cells chronically producing virions (line D). Similarly, PCR fragments of various sizes were apparent in CEM cells exposed to HIV-1 and treated with 13B8-2 (line C). No radioactive PCR fragment was obtained using samples prepared from uninfected CEM cells (line E), pBru plasmid at 10^{11} copies (line F), or quiescent peripheral blood mononuclear cells exposed to HIV-1 (line A).

PCR amplification fragments of approximate size 1–2.5 kb, obtained from HIV-1-infected cells treated with 13B8-2 mAb, were cloned into puc 19 plasmids and several inserts were sequenced. One insert (puc19 # 1) had a sequence identical to the previously published HIV-1Lai LTR sequence having at its 5′ end a TG flanked by another sequence (Figure 5B). Comparison of the flanking sequence with sequences from the NBRF data base indicated that it was not likely to originate from plasmid or HIV and is thus probably a chromosome sequence. Several other inserts were sequenced, but did not differ from puc19 # 1 (data not shown).

13B8-2 mAb inhibits HIV gene transcription

To determine whether the lack of virus production from infected cells treated with 13B8-2 mAb was due to a lack of viral RNA expression, we analyzed HIV-1Lai RNA transcription in infected cells on day 3 following exposure to virus. We used the M667/VPR2 oligonucleotide primer pair, designed to flank the common splice donor and acceptor sites of the Env, Tat and Rev genes (see Figure 2). Spliced mRNA was found in cells exposed to HIV-1 and cultured in the absence of mAb (Figure 6A, line 9) and in cells cultured in the presence of OKT4 (line 11), but not in cells treated with 13B8-2 (line 13). This result suggests that integrated HIV-1 proviruses are transcriptionally inactivated by 13B8-2 treatment, accounting for the lack of production of progeny virions by those cells.

This inhibition of transcription was further analyzed using a CAT assay. CEM cells were transfected by electroporation with the PM-183 plasmid containing the CAT gene under control of the HIV-1Lai LTR promoter. Basal CAT gene transcription was evaluated by cultivating the cells in medium

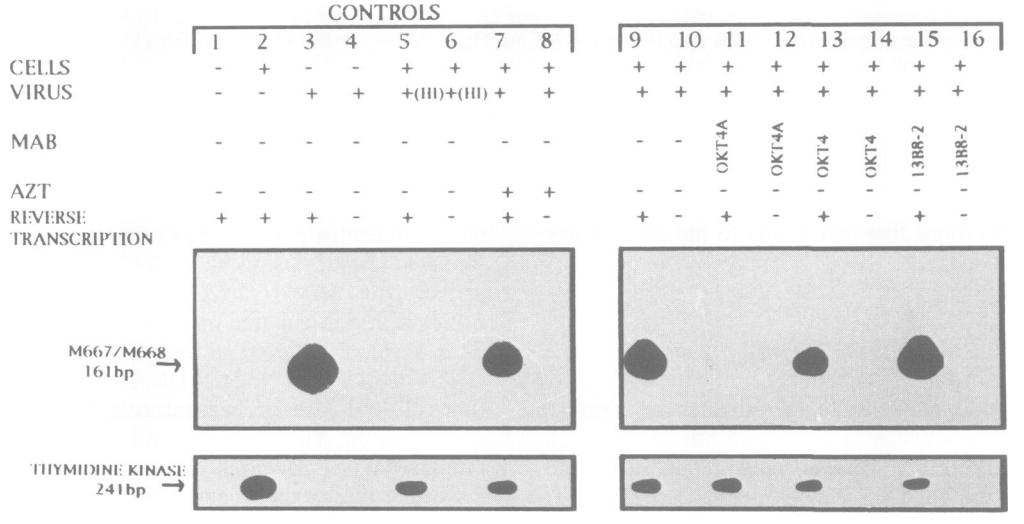

Fig. 3. PCR analysis of HIV-1 full-length RNA in HIV-1-exposed CEM cells. CEM cells were treated with OKT4A, AZT or medium alone, then exposed to HIV for 30 min, extensively washed and cultured for 30 min in medium containing anti-CD4 mAb, AZT or in medium alone, then RNAs were extracted and detected by M667/M668 PCR amplification of AMV-retrotranscribed HIV RNA into DNA as described in Materials and methods. Amplified fragments were hybridized to an α-^{32}P-labeled HIV-1Lai probe, and visualized by autoradiography. An autoradiogram of PCR amplification of retrotranscribed thymidine kinase (TK) RNA, hybridized with α-^{32}P-labeled TK probe, is shown as a control. The different controls specifically prepared for PCR consisted of culture medium alone treated as the extracted samples (line 1), virus-free CEM cells (line 2), HIV-1Lai particles (lines 3 and 4), cells exposed to heat-inactivated HIV-1Lai (lines 5 and 6), and cells exposed to HIV-1Lai particles and treated with AZT (lines 7 and 8). The assay was performed starting from cells exposed to HIV-1Lai and cultured in the absence of inhibitor (lines 9 and 10) or in the presence of anti-CD4 mAb OKT4A (lines 11 and 12), OKT4 (lines 13 and 14), or 13B8-2 (lines 15 and 16). Lines 4, 6, 8, 10, 12 14 and 16 correspond to control samples in which RNAs were not submitted to AMV reverse transcription in order to ensure that no HIV-1 DNA contamination was present in the sample before reverse transcription.

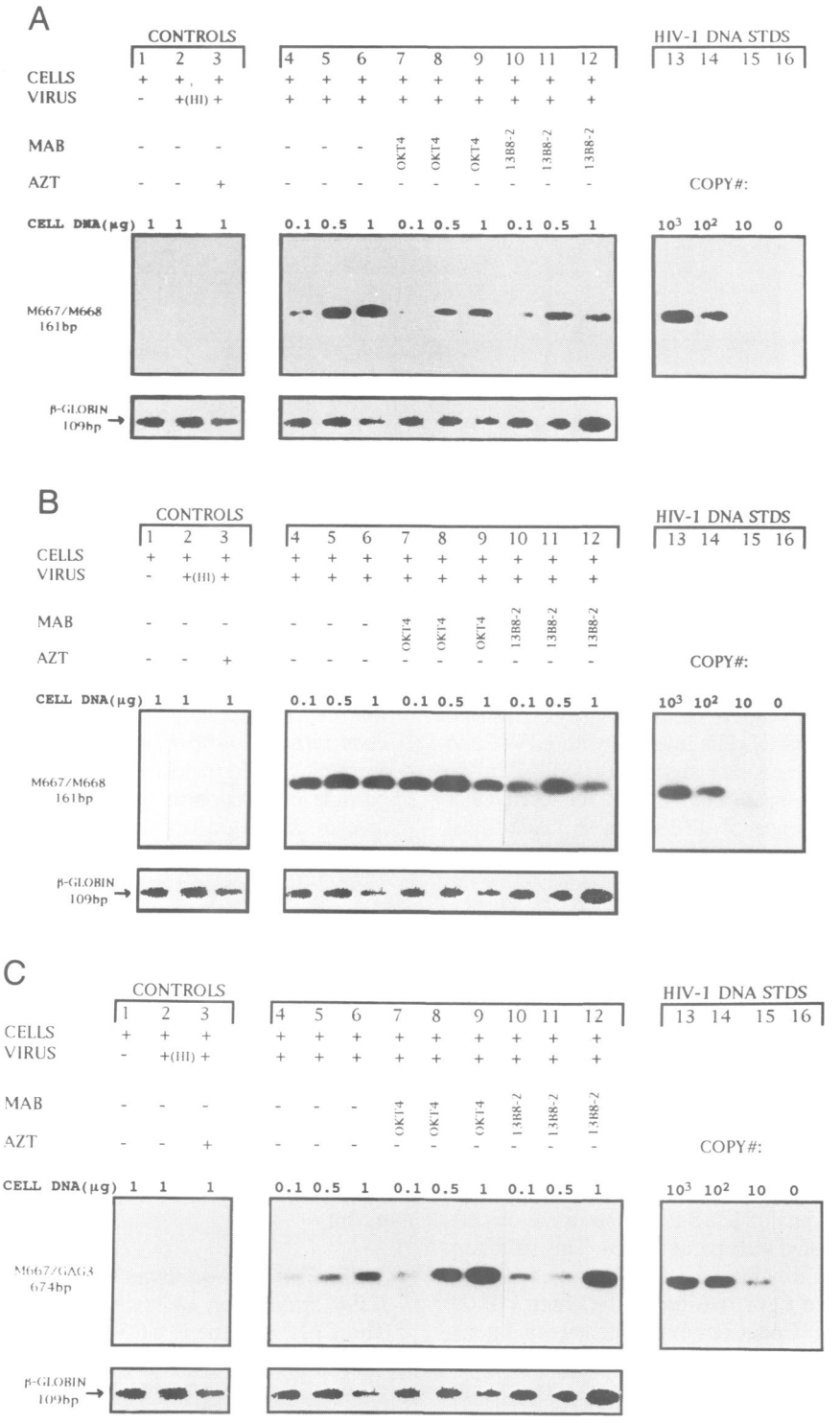

Fig. 4. PCR analysis of viral DNA in HIV-exposed CEM cells. CEM cells were exposed to HIV-1Lai, extensively washed and cultured in the presence of either anti-CD4 mAb 13B8-2 or OKT4, or medium alone. HIV-1 DNA was monitored by PCR analysis performed on total DNA extracts prepared from cells at different times (6 and 24 h) following viral exposure. The controls for PCR were virus-free CEM cells (line 1), cells exposed to heat-inactivated HIV-1Lai (line 2), and cells exposed to HIV-1Lai particles and treated with AZT (line 3). The assay was performed on various amounts of DNA (0.1, 0.5 and 1 µg, respectively) extracted from cells exposed to HIV-1Lai and cultured in the absence of inhibitor (lines 4–6) or in the presence of anti-CD4 mAb OKT4 (lines 7–9) or 13B8-2 (lines 10–12). A control for estimation of the amount of DNA consisted of dilutions of pBRU plasmid containing 1×10^3, 1×10^2 or 1×10^1 copies of the HIV-1Lai genome (lines 13, 14 and 15, respectively), and a control without viral DNA (line 16). The amplified products were electrophoresed, blotted and hybridized with a radiolabeled HIV-1 probe. Labeled viral DNA products were visualized by autoradiography. Product of PCR amplification using β-globin I/β-globin II oligonucleotide primer pair, hybridized with ^{32}P-labeled β-globin probe, is shown as a control. (**A**) Autoradiogram of PCR amplification fragments obtained using the M667/M668 pair on total DNA extract prepared from cells cultured for 6 h following viral exposure. (**B**) Autoradiogram of PCR amplification fragments obtained using the M667/M668 pair on total DNA extract prepared from cells cultured for 24 h following viral exposure. (**C**) Analysis of PCR amplification fragments obtained using the M667/GAG3 oligonucleotide pair on total DNA extract prepared from cell cultures 24 h following viral exposure.

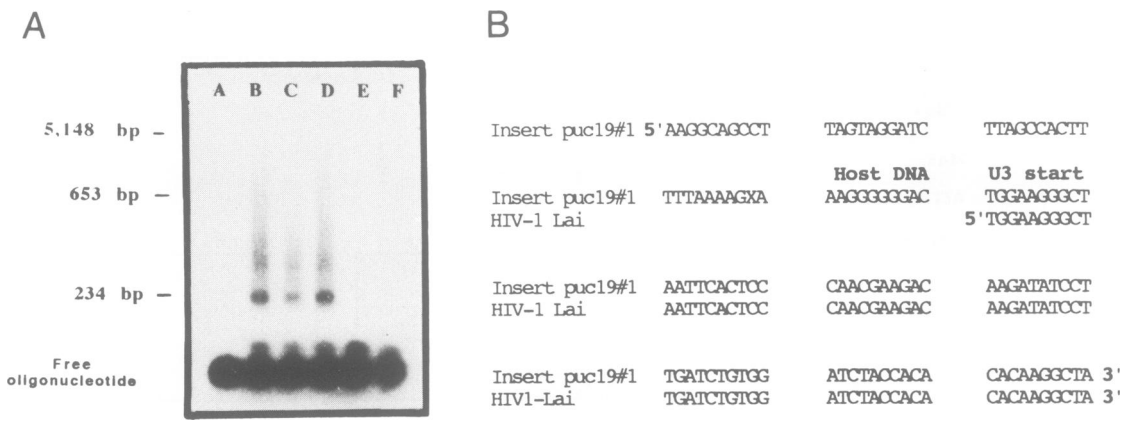

Fig. 5. PCR analysis of integrated HIV-1 DNA in CEM cells. (**A**) High molecular weight DNA was extracted from resting T cells exposed to HIV-1 (line A), CEM cells exposed to HIV-1 in the presence of OKT4 (line B) or 13B8-2 mAb (line C), CEM cells chronically producing HIV-1 (line D) or uninfected CEM cells (line E) and submitted to PCR using the *Alu*I/5′ end-labeled M668 oligonucleotide primer pair in order to amplify hybrid cellular−HIV-1 DNA sequences. PCR amplification products were electrophoresed, transferred onto nylon sheet, and analyzed by autoradiography. PCR of pBru at 10^{11} copies (line F) is shown as a control. (**B**) The *Alu*I/M668 1−2.5 kb PCR amplification products were cloned into puc 19 plasmid and several inserts from the recombinant plasmids were sequenced according to the chain terminating inhibitor method. The lower sequence corresponds to the published HIV-1Lai sequence (Wain-Hobson *et al.*, 1985). The upper sequence represents one partial insert that runs through the HIV-1 5′ LTR of the integrated provirus up to its flanking 5′ sequence.

alone, whereas phorbol myristate acetate (PMA)-induced transcription served as a positive control. Assays consisted of cells treated with 13B8-2, cells infected with HIV-1 and cultured either in the presence or absence of 13B8-2 mAb, and cells exposed to heat-inactivated virus and cultured either in the presence or absence of 13B8-2 mAb. Additional control experiments consisted of CEM cells transfected with the PM-182 plasmid containing the CAT gene driven by the SV40 promoter (control for constitutive expression of CAT gene) and treated under the same conditions. As shown in Figure 6B, the addition of PMA to cells transfected with PM-183 (B) resulted in a 50-fold increase in CAT synthesis relative to untreated cells (A). CAT expression was also induced following infection with HIV-1 (D) or even following contact with viral antigen of heat-inactivated particles (F). However, when HIV-1-infected cells were subsequently treated with 13B8-2 mAb (E), CAT expression was reduced to the level seen in A, suggesting a blockade of CAT gene expression consecutive to 13B8-2 mAb treatment. A similar effect of 13B8-2 was observed on cells exposed to heat-inactivated viral particles (G). This inhibitory effect of 13B8-2 mAb treatment on CAT gene expression was not observed when CAT was under the control of the SV40 promoter (E, G). Under our experimental conditions, 13B8-2 mAb treatment did not inhibit the LTR-driven CAT gene expression induced by PMA (data not shown). Co-transfection experiments were also performed to study the effect of 13B8-2 mAb treatment on LTR-driven CAT gene expression induced by HIV-1Lai Tat. As shown in Figure 6B (H, I), 13B8-2 mAb treatment strongly reduced LTR-driven CAT gene expression induced by Tat. This inhibitory effect of 13B8-2 mAb treatment on CAT gene expression was not observed when CAT was under the control of the SV40 promoter (H, I). We conclude that 13B8-2 mAb treatment itself is sufficient to inhibit CAT gene expression when placed under the HIV-1Lai LTR promoter, possibly by modulating DNA-binding proteins specific for the HIV-1 LTR enhancer.

13B8-2 mAb acts in a dose-dependent manner and restores cell-surface expression of CD4 antigen on cells infected with HIV

We first studied the capacity of 13B8-2 to inhibit HIV-1Lai particle production at concentrations below the saturating level of 2.5 μg/ml. Complete inhibition of viral particle production was observed following treatment with 10 μg/ml 13B8-2, but decreases in the level of particle production were also observed at concentrations of 13B8-2 as low as 0.1 μg/ml (Figure 7).

Since HIV infection is known to induce downregulation of CD4 at the surface of infected cells, we tested whether 13B8-2 treatment may restore CD4 molecule expression at the surface of HIV-1 chronically infected cells after 3 days of treatment with various concentrations of mAb. As shown in Figure 8, treatment with 0.01 μg/ml was sufficient to observe an increase of CD4 expression. CD4 expression was of the same order of magnitude as that of uninfected CEM cells when treatment was performed at concentrations ≥1 μg/ml.

13B8-2 mAb modulates cell-surface expression of IL2-R antigen on CEM cells infected with HIV

The CEM cell line is CD3 and IL2-R negative (where IL2 is interleukin 2), and its growth is IL2 independent (Hillman *et al.*, 1992). However, we have observed a surface expression of IL2-R following HIV-1 infection of CEM cells. Although 13B8-2 mAb treatment affected neither cell viability (scored under photonic microscope by trypan blue evaluation) nor cell division rate (scored under photonic microscope by cell counting) (data not shown), we studied whether it could affect expression of endogenous T cell genes by looking for modulation of cell-surface expression of IL2-R induced following HIV-1 infection. As shown in Figure 9, CEM cells which were IL2-R negative (A) expressed IL2-R following HIV-1 infection (C) at a level similar to that seen with the control MT2 cells (B). However, this antigen expression is modulated by 13B8-2 mAb treatment (D).

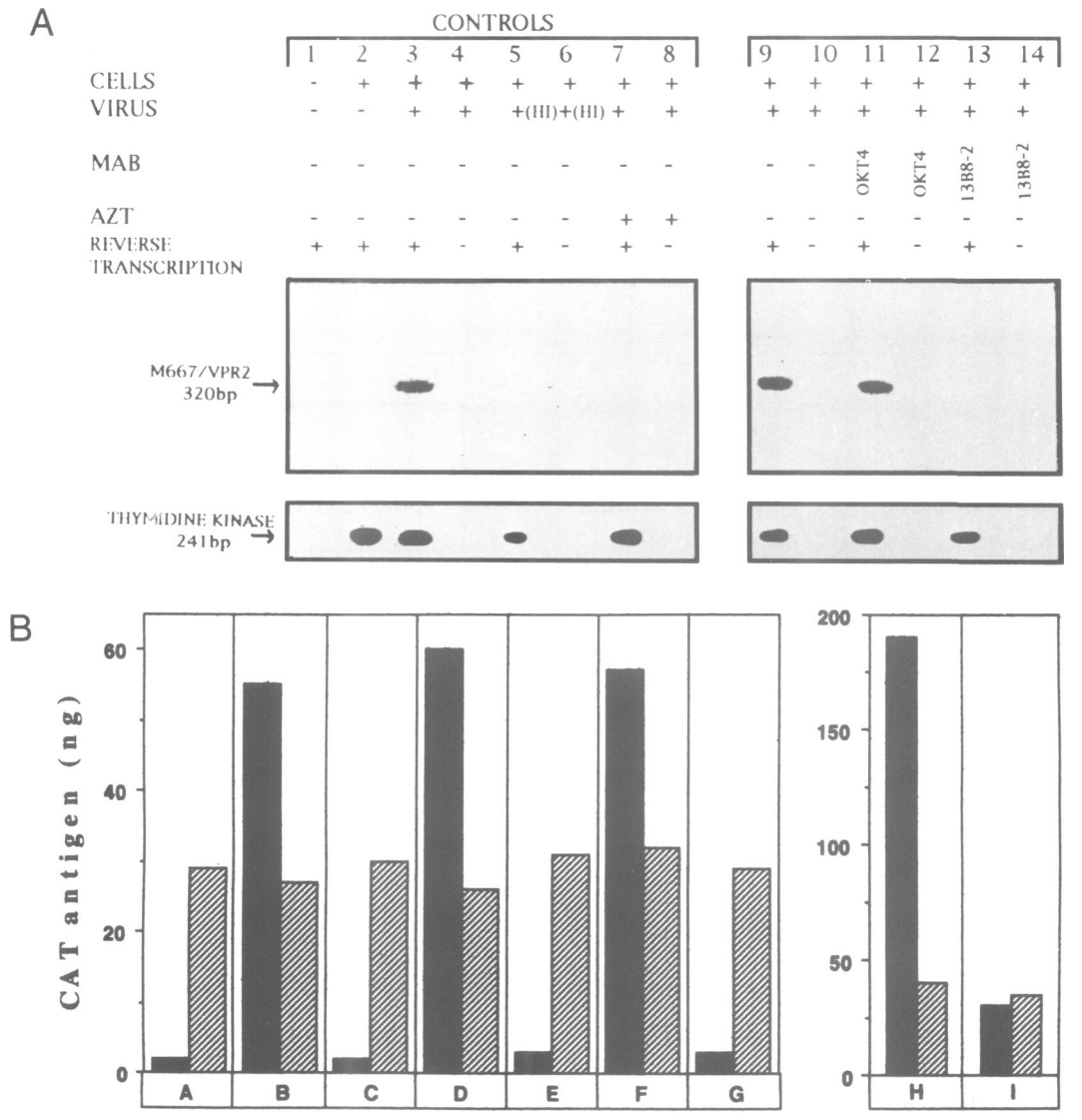

Fig. 6. Analysis of HIV-1 gene transcription. (**A**) PCR analysis of HIV-1 spliced RNA in HIV-1 exposed cells. RNAs were extracted and detected by M667/VPR2 pair PCR amplification of AMV-retrotranscribed HIV-RNA into DNA as described in Materials and methods. Amplified fragments were hybridized to an α-^{32}P-labeled HIV-1Lai probe, and visualized by autoradiography. An autoradiogram of PCR amplification of retrotranscribed thymidine kinase (TK) RNA, hybridized with α-^{32}P-labeled TK probe, is shown as a control. The different controls specifically prepared for PCR were culture medium alone treated like the extracted samples (line 1), virus-free CEM cells (line 2), HIV-1Lai chronically infected CEM cells (lines 3 and 4), cells exposed to heat-inactivated HIV-1Lai (lines 5 and 6), and cells exposed to HIV-1Lai particles and treated with AZT (lines 7 and 8). The assay was performed starting from cells exposed to HIV-1Lai and cultured in the absence of inhibitor (lines 9 and 10) or in the presence of anti-CD4 mAb OKT4 (lines 11 and 12), or 13B8-2 (lines 13 and 14). Lines 4, 6, 8, 10, 12 and 14 correspond to control samples in which RNA were not submitted to AMV reverse transcription in order to ensure that no HIV-1 DNA contamination was present in the sample before reverse transcription. (**B**) CAT assay. CEM cells were transfected with either HIV-1 LTR-CAT (■) or SV40-CAT (▨) at 20 μg DNA/1 × 10^7 cells by electroporation. CAT assays were conducted using a CAT antigen capture assay (Boehringer) with an internal standard that allows conversion of absorbance values into amount of antigen. The different assays consisted of untreated transfected cells (**A**), CEM cells cultured in medium containing 10 ng/ml PMA (**B**), CEM cells cultured in medium containing 10 μg/ml 13B8-2 (**C**), CEM cells infected with HIV at 100 $TCID_{50}$ (**D**), CEM cells infected with HIV at 100 $TCID_{50}$ and cultured in medium containing 10 μg/ml 13B8-2 (**E**), CEM cells exposed to heat-inactivated virus (**F**), CEM cells exposed to heat-inactivated virus and cultured in medium containing 10 μg/ml 13B8-2 (**G**). Co-transfection experiments: CEM cells were transfected with either HIV-1 LTR-CAT and SV40-Tat (■) or SV40-CAT and SV40-Tat (▨) at 40 μg DNA/1 × 10^7 cells by electroporation and cultured in the absence of mAb (**H**) or in medium containing 10 μg/ml 13B8-2 (**I**). CAT assays were conducted as described above.

These results demonstrate that 13B8-2 mAb treatment may modulate the expression of IL2-R gene induced following HIV-1 infection.

13B8-2 mAb treatment is efficient on cells infected with either type 1 or type 2 strains of HIV

Since 13B8-2 mAb has therapeutic potential for AIDS and because a pilot phase I study using 13B8-2 mAb has already been performed in AIDS patients to evaluate the *in vivo* effect of molecules binding the Ig CDR3-like region of CD4 and the consequences of such a treatment on the pathogenesis of AIDS (Dhiver *et al.*, 1989), we assayed 13B8-2 for inhibition of virus particle production by cells exposed to different types and strains of HIV, and for interference with viral production by cells chronically producing viral particles. As shown in Figure 10A, 13B8-2 mAb also

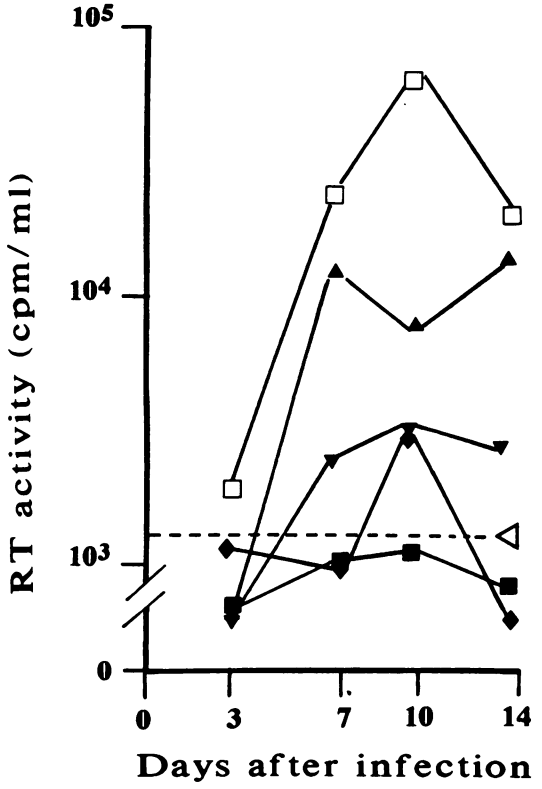

Fig. 7. Dose-dependent effects of 13B8-2. CEM cells were exposed to 100 $TCID_{50}$ HIV-1Lai and cultured in medium alone (□), or medium supplemented with anti-CD4 mAb 13B8-2 at 0.01 μg/ml (▲), 0.1 μg/ml (▼), 1 μg/ml (◆) or 10 μg/ml (■), and RT activity was evaluated in the cell-free culture supernatant of infected cells.

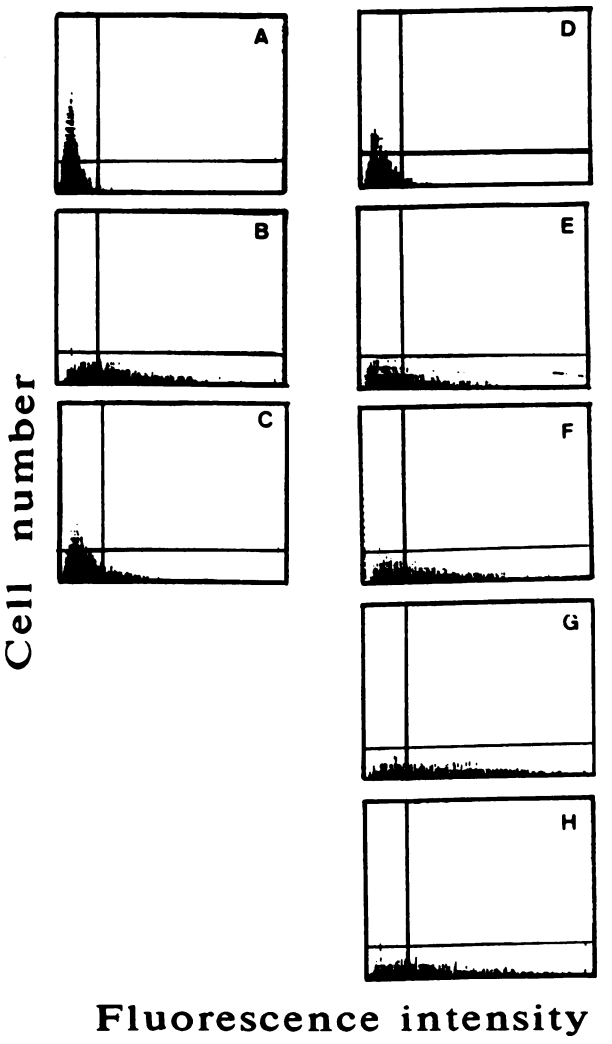

Fig. 8. 13B8-2-induced modulation of CD4 cell-surface expression in HIV-1-infected cells. HIV-1 productively infected CEM cells were cultured for 7 days, and then treated for 3 days with various concentrations of anti-CD4 mAb 13B8-2 (IgG1) used at 0.01 μg/ml (**E**), 0.1 μg/ml (**F**), 1 μg/ml (**G**), or 10 μg/ml (**H**). Controls consisted of CEM cells (**B**) and HIV-1 infected CEM cells cultured for 7 days (**C**) or 10 days (**D**) in the absence of mAb. Cell-surface CD4 was detected by OKT4 mAb (IgG2b) and a fluoresceinated rat anti-mouse (RAM)-IgG2b specific probe. The direct binding of the probe on CEM cells (background) is shown in (**A**). Fluorescence intensity was recorded using the linear channels 0–1024.

prevents viral particle production by cells exposed to HIV-1SF2, HIV-1Eli and HIV-2Rod on day 10 post-infection, whereas the presence of virus particles was detected on day 3 post-infection in culture supernatants of cells untreated with 13B8-2 after viral exposure. The presence of viral DNA in cells exposed to all HIV-1 strains and treated with 13B8-2 was demonstrated by PCR (Figure 10B).

To evaluate the significance of 13B8-2 mAb treatment on cells infected with HIV-1 and chronically producing virus particles, cells were exposed to HIV-1 and treated with 13B8-2 mAb only after reverse transcriptase activity in cell culture supernatants had reached a plateau value. As shown in Figure 11, under such conditions 13B8-2 mAb treatment had nearly immediate effects on virus production, which was completely blocked after a few days of treatment. Conversely, we also demonstrated that the effect of 13B8-2 on HIV production was reversible; when 13B8-2 mAb treatment was stopped, RT activity was evidenced in the culture supernatants of previously non-producing HIV-1-infected cells.

Discussion

The main purpose of this study was to investigate the stage at which the HIV-1 intracellular replicative life cycle is blocked following cell-surface interaction between the anti-CD4 mAb 13B8-2 and the CD4 receptor. We found that in the presence of 13B8-2 mAb the virus entered the cell, and its genome was retrotranscribed into double-stranded DNA and integrated into the host genome. However, the integrated provirus(es) was transcriptionally inactive. Moreover, we found that 13B8-2 mAb blocked both HIV-1 and HIV-2 isolates.

The results of our study, and particularly the HIV-1Lai LTR-driven CAT gene expression assay, strongly favor the hypothesis that the antiviral effect of 13B8-2 we describe is mediated through a DNA- or RNA-binding protein(s) that interacts with the HIV LTR U3/R region. It has been widely documented that HIV has adapted itself to the cell cycle and activation state of the cellular host by incorporating in its LTR regulatory elements capable of binding a variety of cellular DNA- and RNA-binding proteins that are normally involved in the regulation of gene expression (Rosen *et al.*, 1985; Starcich *et al.*, 1985; Franza *et al.*, 1987, 1988; Nabel and Baltimore, 1987; Tong-Starksen *et al.*, 1987, 1989; Gowda *et al.*, 1989; Lewis *et al.*, 1992). Transcription of

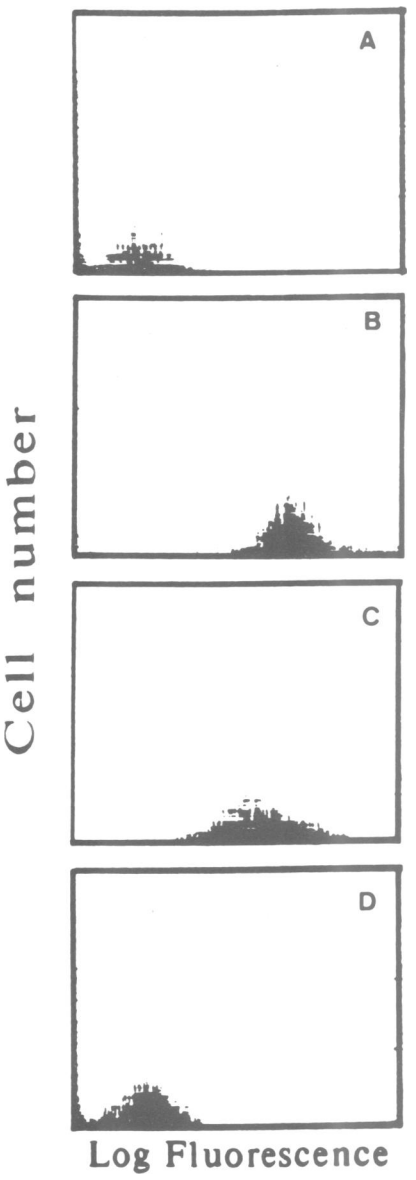

Fig. 9. 13B8-2 induced modulation of IL2-R cell-surface expression in HIV-1-infected cells. HIV-1-infected CEM cells were cultured for 7 days in the absence (**C**) or presence (**D**) of anti-CD4 mAb 13B8-2 at 10 μg/ml. Cell-surface expression of IL2-R was detected by a FITC-labeled anti-CD25 mAb. Controls consisted of CEM (**A**) and MT2 (**B**) cells. Fluorescence data were recorded in the Log mode.

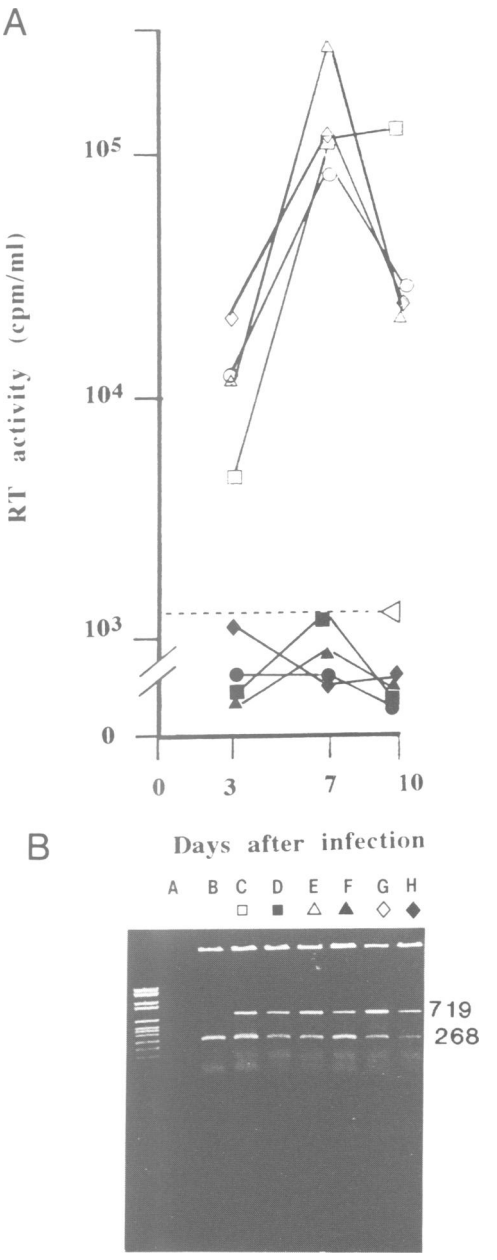

Fig. 10. Effect of 13B8-2 treatment on different HIV isolates. (**A**) Inhibition of viral particle production by 13B8-2 mAb added to the culture medium of HIV-1- or HIV-2-exposed CD4+ cells. CEM cells were exposed to 100 $TCID_{50}$ of HIV-1Lai (□, ■), HIV-1SF2 (△, ▲), HIV-1Eli (◇, ◆) or HIV-2Rod (○, ●) and cultured in the presence or absence of anti-CD4 mAb. Viral production was monitored by measuring the RT activity in cell-free culture supernatant of cells infected and cultured in the absence of inhibitor (open symbols) or cultured in the presence of 13B8-2 (closed symbols). (**B**) The presence of HIV DNA in cells exposed to HIV-1Lai (lanes C and D), HIV-1SF2 (lanes E and F), or HIV-1Eli (lanes G and H) cultured in the absence of inhibitor (lanes C, E and G; see also the open symbols and the legend to Figure 8A) or in the presence of 13B8-2 (closed symbols) was studied on day 7 after viral exposure by PCR using GAG1/GAG2 oligonucleotide primer pair. The amplified products were 719 bp fragments and were revealed under UV illumination after ethidium bromide staining. Products of PCR amplification using β-globin III/β-globin IV oligonucleotide primer pair (268 bp fragments amplification products) are shown as a control. PCR performed in the absence of cells or in the presence of uninfected CEM cells (lanes A and B, respectively) is also shown.

the HIV-1 provirus may therefore be influenced by the action of constitutively expressed cellular DNA-binding factors, such as Sp-1 and TFIID, inducible cellular DNA-binding factors, such as NF-κB and NFAT-1, or even RNA-binding factors, such as TRP-2 and TRP-185 (reviewed by Gaynor, 1992). Since we found that 13B8-2 mAb treatment blocked HIV-1 LTR-driven CAT gene expression following exposure of transfected cells to heat-inactivated virus (a system expected to be Tat-free), and because previous studies have shown NF-κB to be an important factor in HIV-1 gene expression in systems in which Tat was not present (Leonard *et al.*, 1989), we first searched for a putative role of NFκB in the inhibitory effect we describe using an electrophoretic mobility shift assay (M.Benkirane, data not shown). However, we found no evidence that NFκB translocation into the nucleus or its binding to the κB unit could be

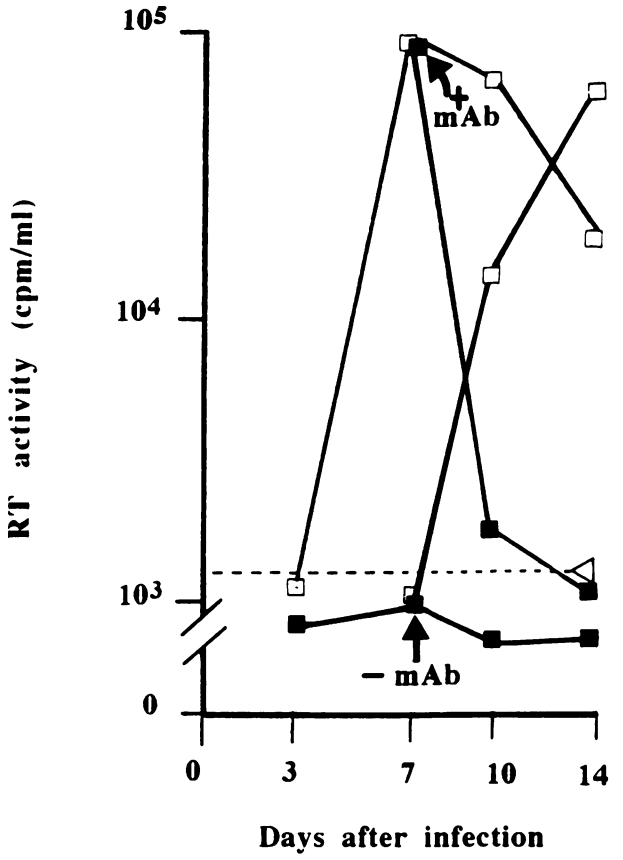

Fig. 11. Effect of 13B8-2 mAb on cells chronically infected with HIV-1Lai. HIV-1 chronically infected CEM cells were treated (■) or not with 13B8-2 (□) at saturating concentrations and RT activity was monitored in cell culture supernatant.

modified following 13B8-2 mAb treatment. Therefore, it is likely that some other DNA- or RNA-binding protein(s) contributes to the transcriptional blockage following 13B8-2 mAb treatment.

One possibility is that after 13B8-2 mAb/CD4 interaction, factor(s) that usually induce HIV gene transcription are inhibited or that factor(s) that block transcription are specifically induced. This has already been proposed as an explanation for proviral latency (Gendelman *et al.*, 1990; Tong-Starksen *et al.*, 1990; Pomerantz *et al.*, 1992). A number of DNA-binding proteins such as AP-1 or COUP, or RNA-binding proteins, such as TRP-2, might play a potential negative regulatory role in HIV-1 gene expression (reviewed by Gaynor, 1992). Recently, it was shown that nuclear expression of human c-Rel markedly represses κB-directed transcription of HIV-1, probably by competition for occupancy of the κB element (Doerre *et al.*, 1993). Interestingly, inhibition of HIV transcription has also been described in the model of HIV and adeno-associated virus type 2 (AAV-2) co-infection. The AAV-2-encoded p^{78rep}/p^{68rep} proteins required for AAV replication repress HIV-1 transcription (Antoni *et al.*, 1991). Moreover, the *rep* gene is able to inhibit the expression of a CAT gene driven by the HIV-1 LTR U3/R region, suggesting that it contains an element responsible for the AAV-2 *rep*-mediated inhibition of HIV transcription (Rittner *et al.*, 1992). Moreover, in the SIV model, two SIVmac strains showed differential transactivation as a result of an 11 base difference in the promoter that creates a third potential Sp-1 site (Anderson and Clements, 1992).

Although at the moment we do not know what putative cellular factor(s) or HIV promoter or repressor sequence(s) are involved in our system, experiments are in progress to understand the molecular mechanisms involved in the downregulation of HIV transcription. We have shown here that 13B8-2 mAb treatment blocks productive infection by a number of reference HIV-1 and HIV-2 laboratory strains, suggesting the putative regulatory unit we are looking for is conserved among the usual isolates. However, it remains possible that this promoter regulation may be under the control of a so far unknown regulatory unit. We have recently derived an *in vitro* variant of HIV-1Lai that is resistant to 13B8-2 mAb treatment (C.David and C.Devaux, unpublished work). This variant should be a useful tool for the identification of this regulatory sequence(s).

In *in vitro* tissue culture experiments using T lymphoblasts, production of virus increases after the cells are exposed to mitogenic lectins and phorbol esters (Harada *et al.*, 1986; Wu *et al.*, 1988). Moreover, deletion of the HIV region (−464 to −252) is capable of suppressing its *in vitro* transcription induced by extracts from Jurkat cells stimulated by PMA (Li *et al.*, 1991). These data and others simply reflect the fact that various cellular signals lead to activation of the *trans*-acting factors that regulate HIV transcriptional activity. Most require mobilization of protein kinase C (PKC) (e.g. NF-κB) which occurs after ligands bind to receptors on the surface of T cells. It has been reported that HIV-1 LTR-directed gene expression by Tat requires PKC (Jakobovits *et al.*, 1990) and reduced susceptibility to HIV-1, has been observed for CEM subclones showing an abnormal PKC signal pathway (Hillman *et al.*, 1992). Other *trans*-acting factors that regulate HIV transcription (e.g. NFAT-1) require the activation of Ca^{2+}-dependent kinases. In parallel to activation of HIV transcription via the PKC pathway, which is common to HIV-1 and HIV-2, it has been reported that the HIV-2 enhancer can be stimulated more efficiently than the HIV-1 enhancer by the Ca^{2+}-mediated signal transduction pathway activated by soluble anti-CD3 (Markovitz *et al.*, 1992). Once we know which DNA- or RNA-binding protein(s) is involved in 13B8-2 mAb-mediated HIV transcriptional inactivation, it will remain to be defined which activation pathway is required for the mobilization of this protein(s).

Our study also raised the question of the role of the CD4 molecule in cell signaling. The CD4 glycoprotein is expressed in a monomeric form at the cell surface and does not possess a kinase domain in its cytoplasmic portion. Its cytoplasmic tail associates with $p56^{lck}$, a member of the src family of tyrosine kinases (Shaw *et al.*, 1989), that may function as its putative signal-transducing element. Highly relevant to this point is the recent observation that tyrosine phosphorylation of several proteins is induced on normal human T cells following 13B8-2 mAb treatment (Hivroz *et al.*, 1993). Interestingly, CD4 mAbs have been reported to be able to inhibit the influx of extracellular Ca^{2+} that occurs after stimulation of the T-cell receptor (TCR)−CD3 complex (Rosoff *et al.*, 1987). However, these observations remain controversial; the Ca^{2+} channel inhibitor verapamil has been described as an inducer of the HIV LTR expression (Hardison *et al.*, 1991). Moreover, mAb directed against the TCR−CD3 complex induced IL2 production as well as HIV LTR-directed gene expression in Jurkat T cells. Addition of cyclosporin A or buffering of intracellular Ca^{2+} did not abolish this LTR-directed gene expression, but did

block IL2 production. In contrast, interference with PKC activation did inhibit both IL2 production and LTR-driven gene expression, indicating that non-mitogenic T-cell activation signals are sufficient to induce HIV transcription (Gruters *et al.*, 1991).

Our results imply that interpretation of the anti-HIV action of a number of CD4 mAbs may have to be reinvestigated, particularly for those mAbs which block productive infection without inhibiting virion binding to target cells (Burkly *et al.*, 1992; Hasunuma *et al.*, 1992; Moore *et al.*, 1992b; Rieber *et al.*, 1992). Indeed, we have recently found another anti-CD4 mAb which does not block virion binding to CD4, but inhibits HIV gene expression (M.Benkirane, P.Corbeau and C.Devaux, unpublished work), indicating that inhibition of HIV transcription by anti-CD4 mAb is not specific to 13B8-2.

Cells infected with HIV show decreased cell-surface expression of CD4 which may contribute to the development of immunodeficiency. This modulation occurs after production of viral antigens by the infected cells. Although reduced steady-state levels of CD4-specific mRNA, Nef gene product-mediated downregulation of cell surface CD4 expression and Vpu-induced degradation of CD4 have been reported, the drop seems to result mainly from the complexing of CD4 antigen with viral envelope gene products (Hoxie *et al.*, 1986; Kawamura *et al.*, 1989; Garcia and Miller, 1991; Chen *et al.*, 1993). Recently, it has been reported that HIV-infected cells treated with a Tat antagonist (Shahabuddin *et al.*, 1992) show an increased expression of free CD4 protein. This is highly relevant to the restoration of cell-surface CD4 expression we observed when HIV-infected cells were treated with 13B8-2 mAb. It could be explained by the inhibition of Env, Nef and Vpu gene product synthesis consecutive to a blockade of transcription.

Every step of the virus life cycle represents a potential target for HIV therapy, and many strategies which could lead to blocking an essential event in the infection process have been developed. These strategies may be classed into two main groups. First, approaches aimed at inhibiting the interaction between the virus particles and the target cells, such as soluble forms of CD4 or anti-gp120 serum antibodies. However, primary HIV isolates show limited susceptibility to soluble CD4 (Moore *et al.*, 1992a) and anti-gp120 are of limited efficacy in that they are virus-type specific (Nara *et al.*, 1990). Second, strategies aimed at inhibiting an intracellular step of the virus replication cycle, such as inhibitors of reverse transcription which have shown clinical efficacy (reviewed by Mitsuya *et al.*, 1991) or viral protease inhibitors (reviewed by Robins and Plattner, 1993). Apart from the problem of escape mutants that are frequently found clinically, there is a limitation in that the drug must enter the target cells to be active. Although it is certainly premature to compare the putative antiviral properties of 13B8-2 mAb with those of known anti-HIV agents inhibiting an intracellular step of the virus replicative cycle, we can at least debate on the *in vitro* properties of these reagents. For the first time, we have shown that a reagent that inhibits an intracellular step of the virus replication cycle does not need to cross the plasma membrane of the HIV-infected cells to be active. This observation opens new possibilities in the search for anti-HIV agents.

Anti-CD4 mAbs have been shown to be able to regulate human T cell function *in vitro* (Wassmer *et al.*, 1985; Blue *et al.*, 1988; Carteron *et al.*, 1989). Consequently, murine and chimeric human/mouse CD4 antibodies have been employed *in vivo* as immunoregulatory tools to influence both experimental immune responses and the clinical expression of a variety of autoimmune diseases, and most recently for blocking of experimental *in vivo* HIV replication in monkeys (Reimann *et al.*, 1993). Treatment with these mAbs has no serious side effects. The question of the harmlessness of using 13B8-2 mAb in patients infected with HIV has already been addressed by others: a pilot phase I study using 13B8-2 mAb treatment in CDC group IV AIDS patients has already been conducted (Dhiver *et al.*, 1989), with relatively encouraging results. New clinical trials using 13B8-2 mAb in HIV-infected patients are currently in progress. In the light of our previous data (Corbeau *et al.*, 1993) showing that 13B8-2 mAb inhibits syncytia formation, and the results presented here showing that 13B8-2 blocks viral production from infected cells and restores their surface expression of CD4, one may expect passive immunotherapy with 13B8-2 during the perinatal period to block maternal–fetal transmission of HIV and may also expect this mAb or a derived molecule to represent a potentially valuable therapeutic agent for AIDS.

Materials and methods

Monoclonal antibody and reagents

Purified anti-CD4 mAb OKT4A (IgG2a) and OKT4 (IgG2b) were purchased from Ortho Diagnostic Systems, Inc. (Raritan, NJ). Purified anti-CD4 mAb IOT4A/13B8-2 (IgG1) was kindly provided by M.Hirn (Immunotech S.A., Marseille, France). Saturating mAb concentrations were previously determined (Corbeau *et al.*, 1993) as 1, 2.5 and 5 μg/ml for OKT4A, 13B8-2 and OKT4, respectively. In all experiments, mAbs were used at concentrations 4-fold higher than that necessary for saturation. The FITC-labeled anti-IL2R (CD25) mAb (IgG2a) was purchased from Coulter (Margency, France). Rabbit anti-mouse (RAM) IgG2b isotype-specific mAb was purchased from Immunotech. AZT was purchased from Boehringer Mannheim (Mannheim, Germany). PMA was purchased from Sigma Chemical Co. (St Louis, MO) and was used at 10 ng/ml in cell cultures.

Oligonucleotides

HIV-1 oligonucleotide primers. M667 (5′-GGCTAACTAGGGAACCCA-CTG-3′ nucleotides 496–516 sense), M668 (5′-TTTCAGGTCCCTGTT-CGGGCGCC-3′ nucleotides 637–656 antisense), VPR2 (5′-CTAGGA-TCTACTGGCTCCATTTC-3′ nucleotides 5836–5859 antisense), GAG1 (5′-GAAGGAGAGAGATGGGTGCGAG-3′ nucleotides 777–803 sense), GAG2 (5′-TATGTCACTTCCCCTTGGTTCTC-3′ nucleotides 1471–1496 antisense), GAG3 (5′-GTCCTGACTGCTGTGTCCTGTG-3′ nucleotides 1148–1170 antisense), LTR3′ (5′-CTTTGTAGTACTCCGGATGC-3′ nucleotides 304–324 antisense).

Cellular oligonucleotide primers. β-globin I (5′-ACACAACTGTGTTC-ACTAGC-3′ nucleotides 14–33 sense), β-globin II (5′-CAACTTCATC-CACGTTCACC-3′ nucleotides 104–123 antisense), β-globin III (5′-CAACTTCATCCACGTTCACC-3′ nucleotides −54 to −73 sense), β-globin IV (5′-GAAGAGCCAAGGACAGGT-3′ nucleotides 176–195 antisense), TK I (5′-GAGTACTCGGGTTCGTGAAC-3′ nucleotides 24–43 sense mRNA), TK II (5′-GGTCATGTGTGCAGAAGCTG-3′ nucleotides 246–265 antisense mRNA), *Alu*I (5′-GCCTCCCAAAGTGCTGGGAT-TA-3′). Oligonucleotides were synthesized by J.-P.Capony (CRBM, Montpellier, France).

Plasmid DNA and transfections

The pBru1 expression vector containing a cloned HIV-1Lai genome (formerly HIV-1Bru strain) was kindly provided by L.Montagnier (Institut Pasteur, Paris, France) and used to prepare HIV-1Lai viral stocks. Briefly, 3×10^6 COS cells in 50 ml flasks (25 cm^2) were transfected with 10 μg of pBru1 using the calcium phosphate co-precipitation method as described by Hirsch *et al.* (1990). Fifteen hours after transfection, the cells were shocked with 20% glycerol in DMEM, rinsed with medium, and co-cultivated with 3×10^6 CEM cells in 6 ml of culture medium (v/v DMEM and RPMI) containing 10% fetal calf serum (FCS). Four days later, CEM cells were harvested and cultivated alone until viruses were detected by RT assay.

Plasmids PM-183 and PM-182 were provided by M.Alizon (ICGM, Paris, France). Briefly, they were derived from the pGEM-4 vector (Promega, Madison, WI) and contain the CAT gene under the control of the HIV-1 LTR and SV40 promoter, respectively. PM-183 (LTR-CAT) contains a *Kpn*I–*Hin*dIII fragment from HIV-1Lai LTR corresponding to the U3/R region located 5′ to CAT and a fragment from SV40 containing the polyadenylation signal (PA-SV40) located 3′ to CAT. PMA 182 (SV40-CAT) contains a *Kpn*I–*Hin*dIII fragment from SV40 containing the enhancer region and replication origin 5′ to CAT and the PA-SV40 3′ to CAT. Plasmid Bg312 HIV-1Lai Tat (SV40-Tat) (Hirsch *et al.*, 1990) was provided by I.Hirsch (INSERM U322, Marseille, France). Transient transfections of cells with 20 μg of the LTR-CAT or SV40-CAT plasmid were carried out by electroporation. Briefly, 1×10^7 cells resuspended in 300 μl of phosphate-buffered saline (PBS) were transfected with 20 μg of the plasmid DNA by an electroshock at 250 V, 960 μF using a Bio-Rad apparatus (Bio-Rad, Richmond, CA). Transfected cells were resuspended at 5×10^5 cells/ml in RPMI containing 20% FCS and antibiotics, and incubated for 18 h at 37°C in 5% CO_2 atmosphere before being used for infection experiments. In some experiments, cells were co-transfected with 20 μg of the LTR-CAT or SV40-CAT plasmid and 20 μg of the SV40-Tat plasmid.

Cells and viruses

The CD4+ lymphoblastoid CEM cell line was obtained from the American Type Culture Collection (Bethesda, MD). The IL2-R-positive HTLV-I-infected MT2 cell line was kindly provided by L.Gazzolo (CNRS UMR30, Lyon, France). Cells were cultured in RPMI 1640 medium supplemented with 1% PSN antibiotic mixture, 1% glutamine (Axcell-Novotec, Lentilly, France) and 10% FCS (ATGC-Biotechnologie, Noisy-Le-Grand, France), to a density of 5×10^5 cells/ml in a 5% CO_2 atmosphere.

Viral stocks were prepared from the chronically infected CEM cell supernatants, as previously described (Corbeau *et al.*, 1991), and kept frozen at −80°C until use. After thawing, 100 μl of these stock viruses (at 10^5 c.p.m./ml) corresponding to 100 $TCID_{50}$ were used for infection assays. In addition to HIV-1Lai, the other viral isolates used in these experiments were HIV-1Eli and HIV-2Rod, provided by L.Montagnier, and HIV-1SF2, provided by H.Holmes (MRC AIDS Reagent Project, Herts, UK). Heat-inactivated virus controls were prepared by incubation for 30 min at 56°C. All viral preparations were treated with 100 U/ml RNase-free DNase for 30 min at room temperature before use.

HIV infection assay

CEM (5×10^5) cells were incubated for 30 min at 4°C in flat-bottomed 96 microwell plates (Costar) with 100 μl of HIV at a concentration of 100 $TCID_{50}$/ml. Thereafter, cells were washed five times and cultured in 24 microwell plates (Costar). The amount of virus produced by CEM cells was monitored twice a week by measuring RT activity in 1 ml of cell-free culture supernatant using a synthetic template primer which permitted the RT to neosynthesize radioactive DNA, as previously described (Corbeau *et al.*, 1991).

CAT assay

Cells transiently transfected with LTR-CAT or SV40-CAT plasmid were exposed to HIV-1 as described above and cultured in the presence or absence of 13B8-2 for 24 h at 37°C, then cells were harvested and centrifuged at 1200 r.p.m. for 3 min. The cell pellet was resuspended in PBS and microfuged at 4°C for 1 min, then washed in TEN buffer [40 mM Tris–HCl (pH 7.4) containing 1 mM EDTA and 150 mM NaCl] and resuspended in 250 mM Tris–HCl (pH 7.8). Cells were disintegrated by repeated (four times) freezing and thawing. After centrifugation, aliquots of the clear supernatant were used for CAT assay. A CAT antigen capture assay (Boehringer Mannheim, Germany) was used to estimate the amount of enzyme contained in the cytoplasmic extracts. An internal control was made available with the kit that allows quantification of the CAT antigen in the extracts. In co-transfection experiments, cells were cultured for 48 h at 37°C in the presence or absence of 13B8-2, then the CAT assay was performed.

Flow cytometry

CD4 modulation assay. CEM cells (1×10^5) were incubated for 30 min at 4°C with saturating concentrations of anti-CD4 mAb OKT4 diluted in PBS containing 0.2% bovine serum albumin (BSA) and 0.1% NaN_2 (PBS–BSA) or medium alone. After washing three times with PBS–BSA, bound mAb was then revealed by addition of 50 μl of a 1/50 dilution of fluoresceinated RAM IgG2b specific mAb (Immunotech S.A.).

CD25 modulation assay. Cells (1×10^5) were incubated for 30 min at 4°C with FITC-labeled anti-CD25 mAb (Coulter).

After 30 min staining, cells were washed with PBS–BSA and fixed in PBS containing 2% formaldehyde and fluorescence intensity was measured on an EPICS PROFILE cytofluorometer (Coulter, Coultronics, Margency, France).

Polymerase chain reaction of DNA and RNA

HIV-1 DNA was monitored by PCR according to the following procedure. Total DNA was extracted from 1×10^6 cells by alkaline lysis and resuspended in 200 μl H_2O. To DNA solution containing various amounts (0.1, 0.5 and 1 μg) was added the amplification mixture: 20 mM Tris–HCl (pH 8.3) containing 120 μM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 0.005% NP40, 0.001% gelatin, 20 pmol of each of the oligonucleotide primers and 2 U of Taq DNA polymerase (Epicentre Technologies, Madison, WI). The amplification reaction was run in a PHC2 thermal cycler (Techne, Cambridge, UK) by submitting the mixture to a first denaturation cycle for 5 min (92°C), followed by primer hybridization for 3 min (at a temperature varying from 50 to 60°C depending on the oligonucleotide primer pair used) and polymerization for 3 min (72°C). Thirty additional cycles were performed under the following conditions: 7 s denaturation, 30 s hybridization, 3 min polymerization (15 min for the last cycle). The amplified products were analyzed by electrophoresis in a 1% agarose gel, blotted for 2 h onto Hybond N^+ membrane (Amersham), and hybridized with α-^{32}P-labeled HIV-1 probe. Labeled viral DNA products were visualized by autoradiography (Kodak X-Omat AR Films). HIV-1 DNA standards used to quantitate viral DNA were derived from dilutions of pBru1 DNA.

PCR detection of retrotranscribed HIV-1 RNA. PCR detection of retrotranscribed HIV RNA was performed according to the previously published procedure (Corbeau *et al.*, 1993). Briefly, total RNA was extracted from 1×10^6 cells. To reduce the amount of HIV-1 DNA originating from lysis, supernatants were treated with RNase-free DNase (Boehringer, 100 U/ml) for 30 min at room temperature in the presence of 10 mM $MgCl_2$ and then phenol extracted before retrotranscription. Samples were resuspended in 40 μl H_2O/0.1% diethyl pyrocarbonate (DEPC). To 20 μl of RNA sample were added 1 U of RNase inhibitor (RNase block II, Stratagene, Ozyme, France), 0.25 U of AMV reverse transcriptase (BRL, Gaithersburg, MD) and 40 pmol of oligo (dT) primer (New England Biolabs, Beverley, MA) in a final volume of 35 μl of reaction buffer [50 mM Tris–HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 500 μM dNTPs]. The primer hybridization was performed at 65°C and slowly cooled to 37°C, then retrotranscription was done at 37°C for 1 h. RNA was then destroyed by adding 0.1 M NaOH solution to this mixture at 50°C for 10 min, followed by neutralization using 0.1 M HCl solution. Neosynthesized HIV DNA was precipitated in 0.3 M sodium acetate (pH 5.2), 10 mM $MgCl_2$ and absolute ethanol for 18 h at −80°C, washed with 70% ethanol and desiccated. Samples were resuspended in 80 μl H_2O and PCR was carried out on 20 μl sample as described above.

DNA sequencing

The *Alu*I/M668 PCR-amplified fragments were phosphorylated using the polynucleotide kinase (Boehringer) and cloned into the *Sma*I/*Sst*I sites of puc19 plasmid. The recombinant plasmids were used to transform *Escherichia coli* JM101 bacteria. Colonies containing HIV-1 sequence were selected, and double-stranded DNA was prepared and sequenced according to the chain terminating inhibitors method (Sanger *et al.*, 1977) using the Sequenase kit (US Biochemical Corp., Cleveland, OH). The 3′ LTR oligonucleotide was used as sequencing primer. The sequencing reactions were analyzed on a 6% acrylamide gel containing 7 M urea.

Acknowledgements

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