

## An Anti-CD4 (CDR3-Loop) Monoclonal Antibody Inhibits Human Immunodeficiency Virus Type 1 Envelope Glycoprotein-Induced Apoptosis

Claire Guillermin,\* Véronique Robert-Hebmann,\* Urszula Hibner,† Michel Hirn,‡ and Christian Devaux\*<sup>1</sup>

\*CRBM/CNRS UPR 1086, Laboratoire Infections Rétrovirales et Signalisation Cellulaire, Institut de Biologie, 4 Boulevard Henri IV, 34060 Montpellier Cedex, France; †CNRS UMR 5535, Institut de Génétique Moléculaire de Montpellier, 34033 Montpellier Cedex 1, France; and ‡Immunotech-Coulter, 13009 Marseille, France

Received March 11, 1998; returned to author for revision April 24, 1998; accepted May 26, 1998

Inhibition of human immunodeficiency virus type 1 (HIV-1)-inducing programmed cell death (PCD) by anti-CD4 monoclonal antibodies (mAbs) was investigated using DNA intercalant YOPRO-1 assay. We found that 13B8.2, an mAb that binds the CDR3-like loop in domain 1 (D1) of CD4, protected infected CEM cell cultures against HIV-1-induced PCD. Protection was not observed using another anti-CD4 mAb (BL4) that binds D1-D2, suggesting that the mechanism involved in cell protection against HIV-1-induced PCD requires engagement of precise CD4 epitopes. Because 13B8.2 is known to inhibit syncytia formation and virus transcription, this mAb could inhibit HIV-1-induced PCD by (1) inhibiting virus gene expression, (2) preventing viral envelope-CD4 interaction, and/or (3) interfering with apoptotic signals. Our data indicated that the absence of enhanced PCD in infected cell cultures treated with 13B8.2 mAb probably was the result of inhibition of HIV-1 replication and virus spread. Moreover, 13B8.2 mAb was found to inhibit PCD mediated by membrane-expressed HIV-1 envelope glycoproteins. Finally, we found that 13B8.2 mAb displayed no protective interference with apoptotic signal induced by Fas, dexamethasone, and serum withdrawal. © 1998 Academic Press

**Key Words:** CD4<sup>+</sup>, signaling, human immunodeficiency virus.

### INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) is the etiological agent of acquired immune deficiency syndrome (AIDS). One of the major characteristics of AIDS is the gradual depletion of CD4<sup>+</sup> T lymphocytes during the development of the disease (Rosenberg and Fauci, 1991). Several mechanisms have been proposed to be responsible for the CD4 cell depletion (Ameisen and Capron, 1991; Laurent-Crawford *et al.*, 1991; Terai *et al.*, 1991; Hovanessian, 1994). Besides destruction of infected cells by HIV-specific cytotoxic lymphocytes (CTLs) and antibody-dependent cellular cytotoxicity (ADCC), these mechanisms include the cytopathic effects (CPE) resulting from the infection of CD4<sup>+</sup> T cells by HIV-1. CPE may be ascribed to HIV-1 envelope glycoprotein 120 (gp120)-induced generation of syncytia, resulting from cell-to-cell transmission of the virus and single-cell killing by induction of programmed cell death (PCD) or apoptosis. Mechanisms involved in HIV-1-induced PCD are not fully understood. PCD can be triggered directly in infected cells by the virus replication, presumably reflecting host defense mechanisms against virus replication, spread, or persistence (Ameisen, 1994; Shen and Shen, 1995). However, PCD also may be mediated indirectly through

priming of uninfected bystander cells to apoptosis by gp120. Experiments aimed at defining the molecular mechanisms involved in HIV-1-induced PCD suggest that it requires viral transcription and cell surface expression of gp120 (Laurent-Crawford *et al.*, 1993; Corbeil and Richman, 1995).

An important goal of HIV research is the identification of molecules inhibitory of HIV-1-induced PCD. During the search for such molecules, anti-CD4 mAb have been reported to inhibit HIV-1-induced PCD (Laurent-Crawford *et al.*, 1993; Hovanessian, 1994; Corbeil and Richman, 1995). However, little is known about the implication of different CD4 epitopes or about the mechanisms by which anti-CD4 mAb that bind outside of the gp120-binding site on CD4 protect cells from apoptosis. In contrast, a large body of data are available concerning the inhibition of the HIV-1 life cycle by anti-CD4 mAb. As a function of the recognized epitope, anti-CD4 mAb will inhibit (1) HIV-1 binding (e.g., anti-CD4 mAb specific for the CDR2-loop in domain 1 of CD4) (Dalglish *et al.*, 1984; Klatzman *et al.*, 1984); (2) fusion, which is likely to require conformational changes in CD4 (Moore *et al.*, 1992); (3) viral transcription, which is controlled by the state of activation of the infected cell (e.g., anti-CD4 mAb specific for the CDR3-loop in domain 1 of CD4) (Benkirane *et al.*, 1993, 1995a); or (4) syncytia formation (e.g., anti-CD4 mAb specific for the CDR2- or CDR3-loop in domain 1 of CD4) (Corbeau *et al.*, 1993). These different

<sup>1</sup> To whom reprint requests should be addressed. Fax: (33) 4-67-60-44-20. E-mail: devaux@sc.univ-montp1.fr.

effects can be explained by the multiple functions of CD4: in addition to acting as a high affinity cell surface receptor for HIV-1 (which binds the CDR2-loop in domain 1 of CD4), CD4 is involved in the postbinding step or steps of the viral replication cycle (Benkirane *et al.*, 1994), as well as in the signal transduction machinery of the cell (Rudd *et al.*, 1988; Veillette *et al.*, 1989). We chose to focus our attention on mAb specific for the CDR3-like region in D1 of CD4. We have previously shown that 13B8.2, a CDR3-like loop-specific mAb, does not inhibit HIV-1 entry into CD4<sup>+</sup> T cells (Corbeau *et al.*, 1993; Benkirane *et al.*, 1993) but rather inhibits HIV-1-induced syncytia formation (Corbeau *et al.*, 1993) and HIV-1 transcription in cells containing an integrated provirus or proviruses (Benkirane *et al.*, 1993, 1995a). Now we have set out to test whether the 13B8.2 mAb, acting on HIV-1 gene expression (including *env* expression), gp120-CD4 binding during cell-to-cell transmission of HIV-1, and cell signaling, inhibits HIV-1-induced PCD.

We report that the CDR3-like loop-specific anti-CD4 13B8.2 mAb protects infected cell culture against HIV-1-induced apoptosis, whereas another anti-CD4 mAb does not.

## RESULTS

### 13B8.2 mAb treatment protects infected cell cultures against HIV-1-mediated cell death

We studied the ability of two anti-CD4 mAbs, 13B8.2 mAb (which binds to the Ig CDR3-like region of the D1 domain of the CD4 molecule) and BL4 mAb (which binds an epitope of the D1-D2 domains of CD4), to protect infected cell cultures against HIV-1-induced cell death. CEM cells were exposed to HIV-1 at 1,000 $\times$  tissue culture infectious dose<sub>50</sub> (TCID<sub>50</sub>)/ml and then cultured in either medium alone or medium supplemented with 13B8.2 mAb or with BL4 mAb at a concentration fourfold greater than necessary for saturation of cell surface CD4. The percentage of dead cells in the culture was estimated by trypan blue exclusion, whereas virus production was evaluated in cell-free culture supernatants by RT activity assay performed twice a week during 2 weeks. HIV-1-induced apoptosis was measured using specific cell staining with the DNA intercalant dye YO-PRO-1. As shown in Figure 1, significant numbers of dead cells appeared in cultures ~3 days after virus production could be detected in culture supernatants of HIV-1-infected cells. The percentage of dead cells in cultures of cells grown in medium alone or in medium supplemented with the BL4 mAb reached a plateau of ~40% on day 6 and day 9 postinfection, respectively. In contrast, the percentage of dead cells in cultures grown in the presence of 13B8.2 mAb was very low and indistinguishable from uninfected CEM cells over 14 days of culture. In agreement with data previously reported by our laboratory (Benkirane *et al.*, 1993, 1995a), viral particles were

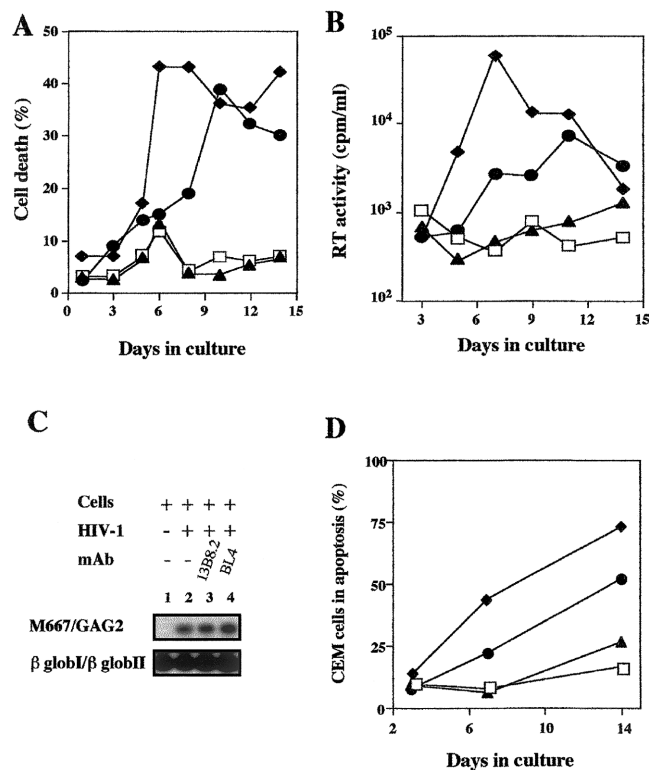


FIG. 1. Effect of anti-CD4 mAb treatment on cell viability and virus production in HIV-1 infected cell cultures. Cells were exposed to 100  $\mu$ l of virus suspension containing 1000  $\times$  TCID<sub>50</sub>/ml HIV-1<sub>LAI</sub>, washed, and cultured in medium alone (◆) or in medium supplemented with anti-CD4 mAb 13B8.2 (▲) or with anti-CD4 mAb BL4 (●) at a concentration fourfold greater than necessary for saturation of cell surface antigens. Uninfected cells are shown as control (□). (A) Viability was evaluated by trypan blue exclusion. (B) Viral production was monitored by measuring reverse transcriptase activity. A representative experiment of four is shown. (C) HIV-1 DNA was monitored by PCR analysis (M667/GAG2 oligonucleotide primer pair) performed on total DNA extracted from CEM cells infected with HIV-1 (lane 2) or CEM cells infected with HIV-1 and treated with anti-CD4 mAb 13B8.2 (lane 3) or BL4 (lane 4) 24 h after virus exposure. Lane 1 represents virus-free CEM cells. Products of PCR amplification using  $\beta$ -globin/ $\beta$ -globin II oligonucleotide primer pair hybridized to an  $\alpha$ -<sup>32</sup>P-labeled  $\beta$ -globin probe are shown as control. (D) The percentage of apoptotic cells in cell cultures was monitored at days 3, 7, and 14 of culture by flow cytometry analysis using the impermeant DNA intercalant YO-PRO-1.

detected in culture supernatants of HIV-1-infected cells grown in medium alone or in medium supplemented with the BL4 mAb, whereas a treatment with the 13B8.2 mAb prevented HIV-1 particle production (Fig.1-B). Moreover, 13B8.2 mAb treatment did not decrease cell proliferation according to our biweekly total cell counts (data not shown; Péléraux *et al.*, 1998).

Although 13B8.2 mAb-treated cells were infected by HIV-1, as demonstrated by polymerase chain reaction (PCR) analysis of HIV-1 DNA performed 24 h after virus exposure (Fig. 1C), 13B8.2 mAb-mediated protection against HIV-1-induced cell death was observed during the 14 days of culture. The percentage of apoptotic cells in HIV-1-infected cells cultured in medium alone or medium supplemented

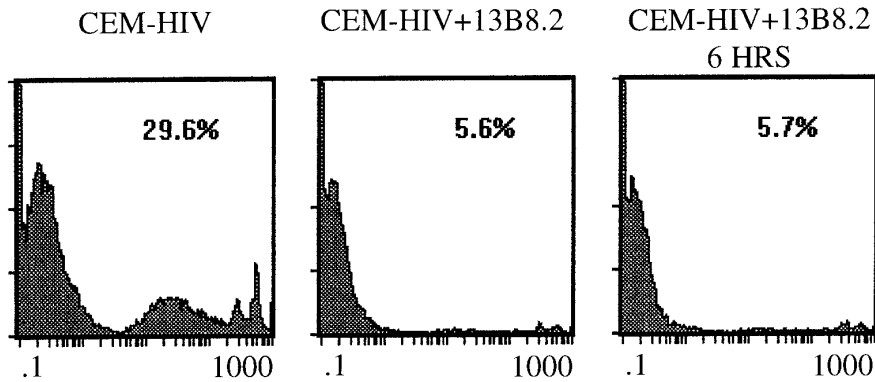


FIG. 2. Effect of 13B8.2 mAb-treatment on HIV-1-induced apoptosis. Cells exposed to HIV-1<sub>LAI</sub> (see legend of Fig. 1 for details) were cultured in medium alone (CEM-HIV) or in medium supplemented with anti-CD4 mAb 13B8.2 immediately after virus exposure (CEM-HIV+13B8.2) or 6 h after virus exposure (CEM-HIV+13B8.2 6 h). The percentage of apoptotic cells in cell cultures was assessed at day 9 of culture by flow cytometry analysis using YOPRO-1.

with anti-CD4 mAb was assessed by flow cytometry analysis on days 3, 7, and 14 postinfection. As shown in Figure 1D, staining by the YOPRO-1 was observed in HIV-1-infected cells cultured in absence of mAb or in the presence of BL4 mAb at 7 days postinfection. After 14 days of culture, most of the cells (72.6% in the culture maintained in medium alone and 51.8% for the culture performed in the presence of BL4) were stained by the YOPRO-1, indicating a major phenomenon of apoptotic cell death. In the uninfected control culture, 16.4% of cells were stained by YOPRO-1, whereas under the same experimental conditions, 26.2% of cells treated by 13B8.2 mAb were stained by the YOPRO-1. Thus, 13B8.2 mAb protected cells against HIV-1-induced apoptotic cell death. Similar results were obtained when 13B8.2 mAb was added to cultures of CEM cells 6 h postinfection (Fig. 2). Under these experimental conditions (in which the early stages of virus replication cycle have occurred), 13B8.2 mAb still protected infected cultures from HIV-1-induced apoptosis (5.7% of cells were stained with YOPRO-1 compared with 29.6% in the control culture). This protective effect of 13B8.2 mAb was confirmed using another assay that consisted of the observation of nucleus condensation and apoptotic bodies formation under fluorescence microscopy after Hoechst nuclear staining (C.G., unpublished observations).

Thus, there was no enhanced cell death in cell cultures treated by the Ig CDR3-like loop-specific 13B8.2 mAb, even though these cells were infected by HIV-1. In contrast, an anti-CD4 mAb directed against a different epitope did not protect cells against HIV-1 infection-associated cell death. However, it should be pointed out that 24 h after virus exposure, the number of infected cells should be low ( $\sim 100/5 \times 10^5$  cells) and virus spread in cell culture should be inhibited by 13B8.2 mAb, which is a known inhibitor of HIV-1 transcription (Benkirane *et al.*, 1993, 1995a), whereas virus spreading occurs in control cultures. Indeed, we reported previously that the amount of viral DNA 7 days after virus exposure was lower in infected CD4 T-cells

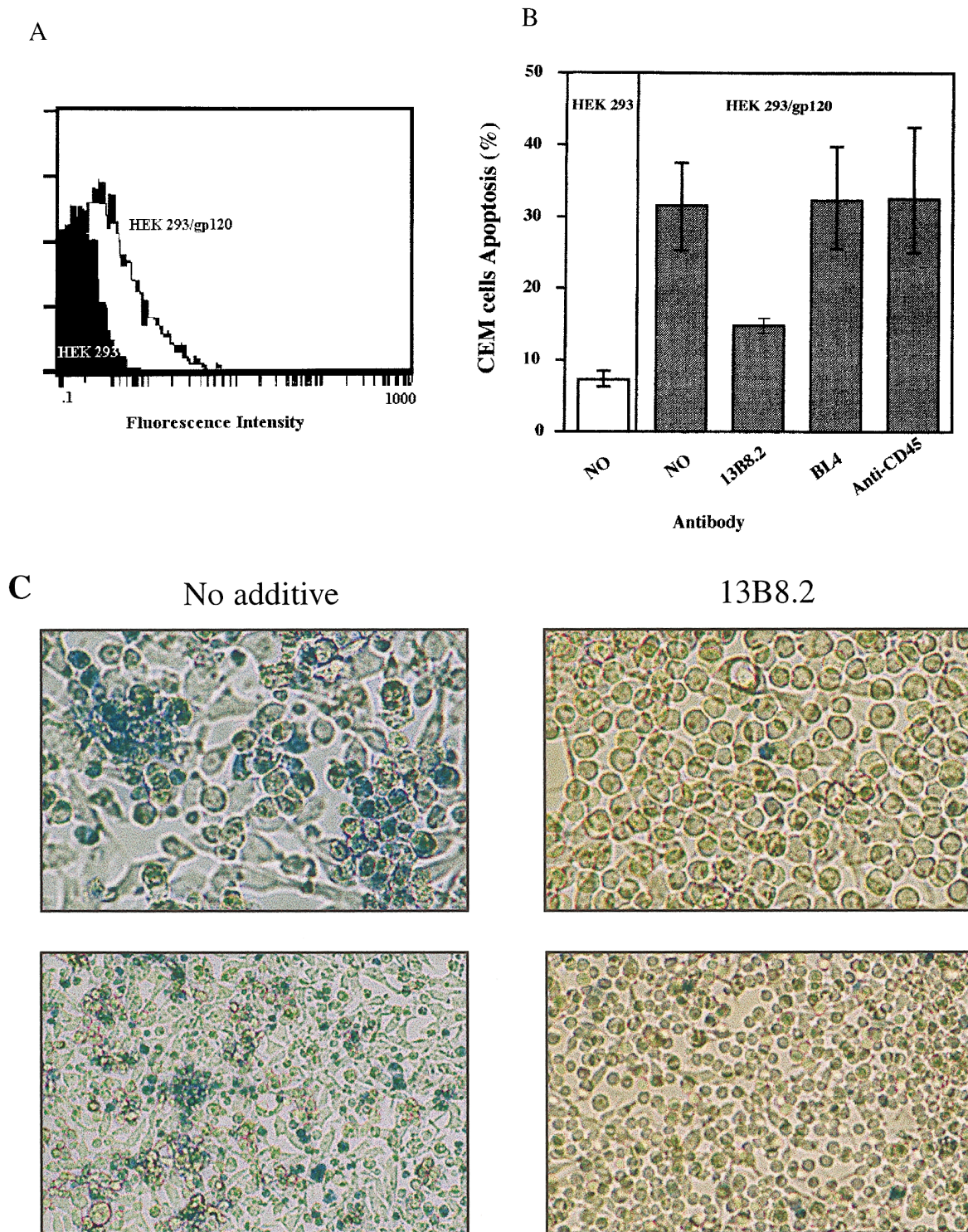
treated with 13B8.2 mAb than in infected control cell cultures (Briant *et al.*, 1996a).

Combined, these data indicate that apoptosis of HIV-1 infected cells was not observed when the cells were cultured in medium containing the Ig CDR3-like loop-specific 13B8.2 mAb, but probably 13B8.2 mAb-mediated inhibition of HIV-1-induced apoptosis resulted from inhibition of HIV-1 transcription leading to inhibition of virus spread.

#### Cell surface-expressed HIV-1 envelope glycoprotein-induced apoptosis is inhibited by 13B8.2 mAb treatment

HIV-1-induced apoptosis is generally thought to require both viral replication and engagement of expressed gp120 with CD4 molecules (Laurent-Crawford, 1993; Corbeil and Richman, 1995). However, membrane-expression of HIV envelope glycoproteins can also trigger apoptosis in CD4<sup>+</sup> cells (Maldarelli *et al.*, 1995). Although the molecular mechanism by which cell surface-expressed gp120 triggers apoptosis in CD4<sup>+</sup> cells is not understood, we designed experiments that were aimed at reproducing this phenomenon and were useful in testing the ability of 13B8.2 mAb to inhibit this process.

We used control human embryonic kidney (HEK) 293 cells and HEK 293 cells transiently transfected by a vector pBRU $\Delta$ gag, which allows HIV-1<sub>LAI</sub> *env* gene expression in the absence of an HIV-1 infection (these cells are hereafter referred to as HEK 293/gp120) to check whether cell surface-expressed HIV-1 envelope glycoprotein can trigger apoptosis in CD4-positive CEM cells during cocultures. As shown in Figure 3-A, HEK 293 cells transiently transfected with pBRU $\Delta$ gag, but not the parental HEK 293 cells, were found to express gp120. As shown in Figure 3B, 31.3% of CEM cells underwent apoptosis in cocultures with HEK 293/gp120 cells, whereas only 7.15% of CEM cells died in cocultures with control HEK 293 cells, indicating that gp120 expressed at the



**FIG. 3.** Effect of coculture between HIV-1 *env*-expressing cells and CEM cells on apoptosis of CEM cells. (A) Expression of HIV-1 envelope glycoprotein at the surface of HEK 293/gp120 cells was assessed by indirect flow cytometry analysis using the anti-gp120 mAb 110.4 and a fluoresceinated GAM probe. The reactivity with untransfected HEK 293 cells is shown as control. (B) HEK 293 or HEK 293/gp120 cells were cocultured with CEM cells either in the absence of mAb (No) or the presence of anti-CD4 mAb (13B8.2 and BL4) or anti-CD45 mAb (9.4). After 3 days of incubation, the nonadherent CEM cells were harvested (the samples contained >99% CD45-positive CEM cells), and the percentage of apoptotic CEM cells in culture was assessed by flow cytometry analysis using YOPRO-1. The results are shown as mean values from triplicate assays (standard deviation is indicated). (C) Photomicrographs illustrating light microscopic observations (magnification, 400-fold for top and 200-fold for bottom) of HEK 293/gp120 adherent cells cocultured with nonadherent (CEM cells for 3 days in the absence of mAb (No additive) or the presence of anti-CD4 mAb (13B8.2). Dead cells were visualized by trypan blue uptake. The difference in color of the pictures (pink in the HIV-1-infected control cell culture and yellow in 13B8.2 mAb-treated culture) reflects the difference in pH of the culture medium, which varies depending on cell proliferation and indicates that 13B8.2 mAb does not appear to have a cytostatic effect.

surface of HEK 293/gp120 cells triggers apoptosis. HEK 293/gp120 cell-induced CEM apoptosis was inhibited by an anti-HIV-1 gp120 V3-loop-specific antibody (C.G., unpublished observations).

Next, we investigated whether 13B8.2 mAb can inhibit cell surface-expressed HIV-1 envelope glycoprotein-induced apoptosis. The percentage of dead cells in untreated cocultures of HEK 293/gp120 with CEM cells was much higher than that in coculture of cells maintained in medium containing 13B8.2 mAb (Fig. 3C). Under the same experimental conditions, when the percentage of apoptotic CEM cells was evaluated by YOPRO-1 assay performed on nonadherent cells (Fig. 3B), 13B8.2 mAb was found to inhibit CEM cell apoptosis (14.7% compared with 31.3% in the control sample). In contrast, neither BL4 anti-CD4 mAb (32.25% apoptosis) nor anti-CD45 mAb (33.5% apoptosis) inhibited HEK 293/gp120-induced apoptosis of CEM cells.

Our data corroborate previous observations (Maldarelli *et al.*, 1995) indicating that gp120 expressed at the surface of "stimulating cells" induces apoptosis in CD4<sup>+</sup> "responding cells." Because our experiments were performed in a model of uninfected cells (HEK 293/gp120) that artificially express gp120, they strongly support the hypothesis of Corbeil and Richman (1995) that engagement of expressed gp120 with CD4 molecules contributes to complete the signalization process in infected cells.

#### 13B8.2 mAb treatment fails to prevent anti-Fas IgM-induced apoptosis or dexamethasone and serum withdrawal-induced apoptosis

Since 13B8.2 mAb acts on T cell activation via CD4 (Benkirane *et al.*, 1993, 1995b; Lemasson *et al.*, 1996), it was tempting to investigate whether this mAb could inhibit signaling pathways which control apoptosis. Cell surface Fas (CD95) expression is strongly induced following HIV-1 infection (Badley *et al.*, 1996), and increase in Fas expression is expected to modify the sensitivity of T cells for Fas-mediated apoptosis *ex-vivo* (Katsikis *et al.*, 1995) and to be related to disease progression and CD4<sup>+</sup> T cell depletion in HIV-1 infected patients (Boudet *et al.*, 1996; Gehri *et al.*, 1995). Therefore, we first focused our attention on the Fas pathway and investigated the ability of 13B8.2 mAb to modulate cell surface expression of CD95. Consistent with data reported in the literature, we found a slight increase in Fas (UB2 epitope) expression 10 days after infection of cells by HIV-1, whereas 13B8.2 mAb treatment maintained surface expression of Fas at a level indistinguishable from that of the uninfected cells (C.G., unpublished observations).

Because 13B8.2 mAb inhibits HIV-1-induced upregulation of Fas, this mAb may influence apoptosis induced through the Fas pathway. Therefore, we next examined the effect of 13B8.2 mAb treatment on CEM cells apoptosis triggered by anti-Fas IgM mAb. Cells were treated

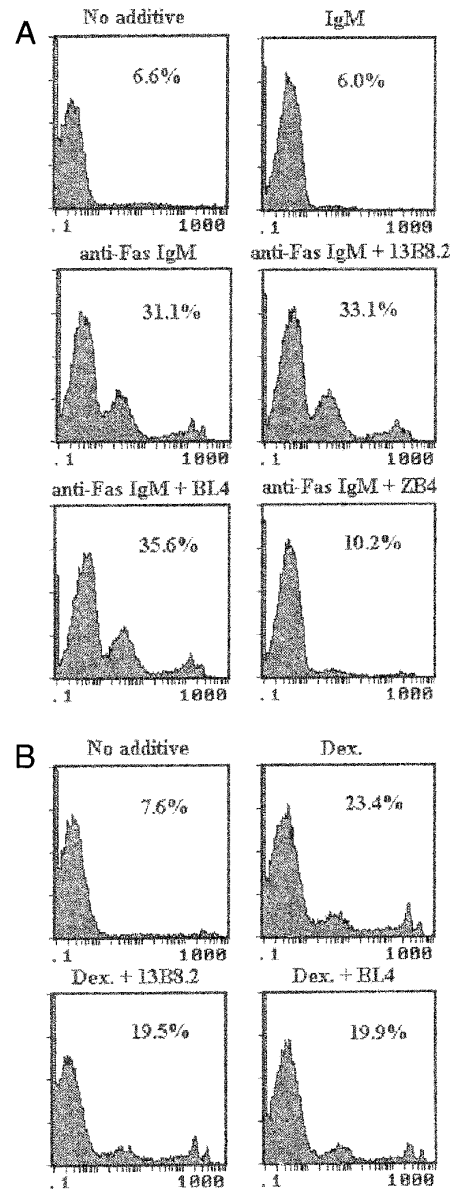


FIG. 4. Effect of anti-CD4 mAb treatment on anti-Fas IgM- and dexamethasone-induced apoptosis. (A) Anti-Fas IgM-induced apoptosis. Cells were cultured in medium alone (no additive) or in medium supplemented with 1  $\mu$ g/ml anti-Fas IgM mAb (CH-11) or with anti-Fas IgM mAb and anti-CD4 mAb (13B8.2 or BL4). The percentage of apoptotic cells in cultures was assessed by flow cytometry analysis using the impermeant DNA intercalant YOPRO-1 at 16 h of culture. Controls consisted of cells treated with ZB4, an antagonist anti-Fas IgM mAb, before anti-Fas IgM mAb treatment and of cells treated with an isotype-matched (IgM) mAb. (B) Dexamethasone ( $10^{-7}$  M)-induced apoptosis. Cells were cultured in medium alone (no additive), in medium supplemented with dexamethasone, or in medium supplemented with dexamethasone and anti-CD4 mAb. The percentage of apoptotic cells in cell cultures was measured as in A at day 3 of culture.

with the CH-11 anti-Fas IgM mAb and then cultured in medium alone or medium supplemented with 13B8.2 or BL4 anti-CD4 mAb and stained by the YOPRO-1. As shown in Figure 4A, apoptosis was induced in cultures of cells treated with CH-11. In contrast, enhanced apoptosis



was not observed with an isotype-matched control Ig and was inhibited by ZB4, an antagonist anti-Fas IgG mAb, which competes with CH-11 for binding to Fas. The presence of 13B8.2 or BL4 anti-CD4 mAb in the culture medium had no significant effect on the observed percentage of apoptotic cells.

To verify that 13B8.2 mAb treatment had no general antiapoptotic effect on the CEM cells, we examined the induction of apoptosis in CEM cells by dexamethasone and by serum withdrawal. Figure 4B illustrates a representative experiment in which apoptosis was induced by dexamethasone. Neither 13B8.2 nor BL4 mAb had a significant effect on CEM cells apoptosis induced by dexamethasone. Similarly, apoptosis induced by serum withdrawal was not inhibited by either 13B8.2 or BL4 mAb treatment (data not shown).

Thus, Ig CDR3-like specific 13B8.2 mAb inhibited HIV-1-induced Fas antigen modulation (probably by inhibiting HIV-1 transcription) but did not inhibit apoptosis induced by anti-Fas IgM stimulation, dexamethasone, or serum withdrawal.

## DISCUSSION

CD4<sup>+</sup> T cells undergo apoptosis when infected by HIV-1. The aim of the present study was to investigate whether anti-CD4 mAb can protect infected cell cultures against HIV-1-induced apoptosis. We show that the treatment of cells with 13B8.2 (a mAb that binds to the CDR3-like loop in D1 of CD4) within the first 6 h postinfection protects the CD4<sup>+</sup>lymphoblastoid CEM cells against HIV-1-induced apoptosis. Moreover, cell surface-expressed HIV-1 envelope glycoprotein-induced apoptosis also was inhibited by 13B8.2 mAb treatment. In contrast, BL4 (another anti-CD4 mAb that binds D1-D2) fails to protect cells against HIV-1-induced and cell surface-expressed HIV-1 envelope glycoprotein-induced PCD.

Several viruses, including retroviruses such as human T-cell leukemia virus type I, counter PCD mechanisms by encoding proteins that inhibit the cellular apoptotic machinery (Levine *et al.*, 1993; Henderson *et al.*, 1993; Chiou *et al.*, 1994; Yamada *et al.*, 1994; Gillet *et al.*, 1995; Afonso *et al.*, 1996). In contrast, HIV-1 is an example of a virus causing a drastic cell depletion by apoptosis (Li *et al.*, 1995; Purvis *et al.*, 1995; Westendorp, 1995). As expected, we found here that HIV-1 infection induces a dramatic PCD in infected cultures. It is worth noting that virus production begins 3 days before significant PCD could be detected in the culture. It is generally admitted that HIV-1 induces activation signals in CD4<sup>+</sup> cells (Benkirane *et al.*, 1994; Chirmule *et al.*, 1995; Merzouki *et al.*, 1995; Popik and Pitha, 1996; Briant *et al.*, 1996). Uncontrolled and chronic immune activation triggered by HIV-1 is probably the primary mechanism responsible for the collapse of the immune system observed in AIDS. This activation process should involve transcription factors

stimulating cell activation and virus replication but also triggering activation-induced apoptosis. Probably, the kinetics of events controlled by the molecular crosstalk between the virus and the host cell should favor the virus replication first, whereas HIV-1-induced PCD should occur at a second stage, once the virus-replicative cycle (at the level of a single cell) is completed. It is worth noting that PCD can be induced by HIV-1 in cells expressing truncated forms of CD4 that lack the cytoplasmic domain (Jacotot *et al.*, 1997; Guillermin *et al.*, 1998), suggesting that delivery of the apoptotic signals probably involves a signal transduction molecule other than CD4. One hypothesis is that the HIV-1 coreceptor CXCR4 maybe implicated in HIV-1-induced PCD.

Several anti-CD4 mAbs (OKT4A and Leu3a, which block gp120 binding to CD4 and inhibit syncytia formation, and OKT4, which demonstrates no ability to inhibit gp120-CD4 interactions), prevent HIV-1-induced PCD when added to the cell culture medium postinfection (Laurent-Crawford *et al.*, 1993; Hovanessian, 1994; Corbeil and Richman, 1995). However, the mechanism by which these mAbs inhibit PCD remains largely unknown. The possibility of rescuing HIV-1-infected cells from PCD by two other anti-CD4 mAbs (13B8.2 and BL4), as well as the possible mechanisms controlling resistance to PCD, were investigated in this study. We found that a treatment of infected cells with 13B8.2 protects the CD4<sup>+</sup> lymphoblastoid CEM cells against HIV-1-induced apoptosis, whereas BL4 shows no such protective effect. We then investigated the mechanisms by which the 13B8.2 mAb protects infected cell cultures from HIV-1-induced apoptosis. One could hypothesize that 13B8.2 inhibits HIV-1-induced PCD by (1) inhibiting virus gene expression, (2) preventing viral envelope-CD4 interaction, and/or (3) interfering with apoptotic signals.

First, we investigated whether 13B8.2 mAb protects infected cells from HIV-1-induced PCD and whether protection results from inhibition of virus gene expression. Indeed, virus gene expression requires cell activation and nuclear translocation of cellular transcription factors acting on the viral promoter. The association of cell activation with apoptosis has been reported in several viral infections. Increased susceptibility to apoptosis of peripheral lymphocytes from an HIV-infected person correlates with an activated phenotype (CD4<sup>+</sup>, HLA-DR<sup>+</sup>, CD38<sup>+</sup>, CD45RO<sup>+</sup>, Fas<sup>+</sup>) (Gougeon *et al.*, 1996). In agreement with previous reports (Benkirane *et al.*, 1994; Borvak *et al.*, 1995; Chirmule *et al.*, 1995; Merzouki *et al.*, 1995; Schmid-Antomarchi *et al.*, 1996), we demonstrated that binding of uninfected HIV-1 (inactivated by heating at 56°C) or gp120-anti-gp120 immune complexes to CD4 molecules of infected resting peripheral blood mononuclear cells was sufficient to activate NF- $\kappa$ B and AP-1 DNA-binding proteins to induce a transition from the G<sub>0</sub>/G<sub>1</sub> to S/G<sub>2</sub>/M stages of the cell cycle, to trigger the cell surface expression of the CD25 activation marker, to

stimulate provirus integration, and to commit cells to virus production (Briant *et al.*, 1996b). We have also previously shown that 13B8.2 mAb blocks activation of MAP kinase (Benkirane *et al.*, 1995b) and NF- $\kappa$ B (Lemasson *et al.*, 1996) induced by HIV-1 binding to CD4 and inhibits HIV-1 transcription (Benkirane *et al.*, 1993, 1995a; Benkirane *et al.*, 1995b; Lemasson *et al.*, 1996; Coudronnière *et al.*, 1998). These results corroborate the observation by Jabado *et al.* (1994) indicating that 13B8.2 mAb inhibits NF- $\kappa$ B activation of interleukin-2 promoter induced by an anti-CD3 mAb plus a protein kinase C activator. Therefore, the mechanism of action of 13B8.2 mAb could correspond, at least in part, to the inhibition of nuclear translocation of NF- $\kappa$ B and the ensuing lack of HIV-1 promoter activation. In our experiments, inhibition of virus gene expression probably accounts for an inhibition of virus spread to uninfected cells, and apoptosis is not induced in the presence of 13B8.2 mAb as the majority of cells are not infected. Moreover, a direct consequence of transcriptional inhibition by 13B8.2 mAb is the lack of gp120 expression at the surface of infected cells. Under these conditions, the mechanism of induction of apoptosis by cell surface-expressed gp120 is inoperative.

Second, we studied whether 13B8.2 mAb, which is known to inhibit HIV-1-mediated syncytia formation (Corbeau *et al.*, 1993), blocks HIV-1-induced apoptosis by preventing viral envelope (expressed at the cell surface) to interact with CD4. We found that HEK 293 cells expressing the HIV-1 envelope glycoprotein trigger CEM cell apoptosis during coculture experiments. Moreover, we demonstrated that 13B8.2 mAb inhibited apoptosis of CEM cells cocultured with HEK293/gp120 cells. This result supports the hypothesis that a direct contact between cell surface gp120 and CD4<sup>+</sup> cells initiates PCD in the CD4<sup>+</sup> population. Obviously, this result does not exclude the possibility that HIV-1-induced PCD involves a signal transduction through the HIV-1 coreceptor CXCR4; indeed there is a possibility that CD4 can indirectly trigger HIV-1-induced PCD by allowing optimal interactions between the gp120-gp41 and the CD4-CXCR4 complexes. This hypothesis proposing that a direct contact between cell surface gp120 and CD4<sup>+</sup> cells initiates PCD could explain why anti-CD4 mAb OKT4A and Leu3a prevent HIV-1-induced PCD when added to the cell culture medium postinfection (Hovanessian, 1994; Corbeil and Richman, 1995); although these mAbs do not act on HIV-1 transcription, they block gp120 binding to CD4. It is worth noting that in contrast to the results obtained using cell surface-expressed gp120, we have not observed induction of PCD by incubating CD4<sup>+</sup> target cells with high concentrations of heat-inactivated HIV-1 (data not shown). This result is suggestive of a specific requirement for cell surface expression of gp120 in HIV-1-induced PCD.

Third, interference with apoptotic signals was ana-

lyzed. Survival signals provided by the cell surface proteins CD2 and CD28 have already been reported to rescue CD4<sup>+</sup> T cells from HIV-gp120-induced apoptosis (Tuosto *et al.*, 1995). Because we have shown previously that 13B8.2 mAb acts on T-cell signaling (Benkirane *et al.*, 1995b; Lemasson *et al.*, 1996; Coudronnière *et al.*, 1998), the possibility that 13B8.2-mediated protection of cells against HIV-1-induced PCD involves a complex mechanism of cell signals preventing apoptosis had to be considered. We found that 13B8.2 mAb does not inhibit anti-Fas IgM mAb-, dexamethasone-, or serum withdrawal-induced apoptosis, suggesting that the effect observed is restricted to HIV-1-induced cell death.

In conclusion, we have shown here that 13B8.2 mAb treatment protects infected cell cultures against HIV-1-induced apoptosis. We found that 13B8.2 mAb-mediated protection against HIV-1-induced PCD parallels inhibition of HIV-1 transcription and virus replication, suggesting that these events are strongly linked. Moreover, 13B8.2 mAb also acts on PCD of CD4<sup>+</sup> cells induced by interaction with cell surface-expressed gp120. Altogether, our results indicate that 13B8.2 mAb treatment inhibits two major steps of HIV-1-replicative cycle that contribute to induce apoptosis.

## MATERIALS AND METHODS

### Monoclonal antibody and reagents

Purified anti-CD4 IOT4A/13B8.2 Ig G1 mAb and IOT4/BL4 IgG2a mAb were prepared by Immunotech-Coulter (Marseille, France). Characterization of these anti-CD4 mAbs was reported previously (Corbeau *et al.*, 1993; Benkirane *et al.*, 1993, 1995a). Anti-CD4 mAbs were used at a concentration fourfold greater than necessary for saturation of cell surface CD4, as previously determined (Corbeau *et al.*, 1993). Anti-Fas (CH-11) IgM mAb, anti-Fas (ZB4) IgM mAb, and FITC-labeled F(ab')<sup>2</sup> goat anti-mouse Ig reagent were purchased from Immunotech. Anti-gp120 (110.4) mAb was provided by Genetic Systems (Seattle, Washington). Anti-CD45 (9.4) mAb was provided by American Type Culture Collection (Bethesda, Maryland). DNA intercalant dye YOPRO-1 was purchased from Molecular Probes (Eugene, Oregon).

### Cells and viruses

The CD4<sup>+</sup> lymphoblastoid CEM cell line was obtained from American Type Culture Collection. Cells were cultured in RPMI 1640 medium supplemented with 1% penicillin-streptomycin antibiotic mixture, 1% Glutamax (Life Technologies, Eragny, France), and 10% fetal calf serum (Life Technologies) to a density of  $5 \times 10^5$  cells/ml in a 5% CO<sub>2</sub> atmosphere. The HEK 293 cell line was provided by P. Boulanger (Institut de Biologie, Montpellier, France). HEK 293/gp120 cells were obtained by transient transfection of HEK 293 cells (using

the calcium phosphate coprecipitation method) with the HIV-1 defective pBRU $\Delta$ gag construct, which allows HIV-1 *env* gene expression. pBRU $\Delta$ gag derives from pBRU3 vector (an HIV-1<sub>LAI</sub> expression vector), kindly provided by L. Montagnier (Institut Pasteur, Paris, France), in which the *PstI*/*Apal* *gag* sequence was deleted. Viral stocks (HIV-1<sub>LAI</sub>) were prepared from chronically infected CEM cell supernatants, as previously described (Corbeau *et al.*, 1990), and were treated for 30 min with 100 U of RNase-free DNase/ml.

### Assay for HIV-1 infection

Cells ( $5 \times 10^5$ ) were incubated for 30 min at 4°C in flat-bottom, 96-microwell plates (Costar, Badhoevedorp, the Netherlands) with 100  $\mu$ l of HIV-1 at a concentration of  $1,000 \times$  TCID<sub>50</sub>/ml. Thereafter, cells were washed five times and cultured in 24-microwell plates (Costar). The amount of HIV-1 produced by CEM cells was monitored twice each week by measuring reverse transcriptase activity in 1 ml of cell-free culture supernatant using a synthetic template primer, as previously described (Corbeau *et al.*, 1990).

### PCR assays

HIV-1-DNA was monitored by PCR according to the previously described procedure (Benkirane *et al.*, 1993). Briefly, total DNA was extracted from  $1 \times 10^6$  cells by alkaline lysis and resuspended into 200  $\mu$ l of H<sub>2</sub>O. The amplification mixture consisting of 20  $\mu$ M Tris-HCl, pH 8.3, containing 120  $\mu$ M concentration of dNTPs, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet P-40, 0.001% gelatin, 20 pmol of each of the oligonucleotide primers M667 and GAG2 (Benkirane *et al.*, 1993), and 2 U of *Taq* DNA polymerase was added to DNA. The amplification reaction was run in a PHC2 thermal cycler (Techne, Cambridge, UK). The amplified products (674-bp fragments) were analyzed by electrophoresis in a 1% agarose gel, blotted for 2 h onto Hybond N<sup>+</sup> membrane (Amersham), and hybridized with  $\alpha$ -<sup>32</sup>P-labeled HIV-1 probe. Labeled viral DNA products were visualized by autoradiography.

### Flow cytometry

Cells ( $1 \times 10^6$ ) were incubated for 60 min at 4°C with saturating concentrations of anti-gp120 mAb or medium alone. After washing three times with PBS containing 0.2% bovine serum albumin, bound mAb was detected by the addition of 50  $\mu$ l of a 1:50 dilution of fluoresceinated goat anti-mouse immunoglobulin (Immunotech). After 60 min of staining, cells were washed with PBS-bovine serum albumin, and fluorescence intensity was measured on a EPICS XL4C cytofluorometer (Coulter, Coultronics, Margency, France). The percentage of apoptotic cells was also assessed by flow cytometry analysis using the impermeant

DNA intercalant YOPRO-1 (10 mM) (EX<sub>max</sub>/EM<sub>max</sub> [nm]=491/509) as described previously (Idziorek *et al.*, 1995).

### Assays for apoptosis induced by membrane-expressed gp120

HEK 293 cells and HEK 293 cells transiently transfected by a vector pBRU $\Delta$ gag (referred to as HEK 293/gp120 cells) were mixed with CD4<sup>+</sup> CEM cells (at a ratio of  $3 \times 10^6$  HEK 293/gp120 cells for  $1 \times 10^6$  CEM cells) and incubated at 37°C for 2 to 3 days either in medium alone or medium containing antibodies at saturating concentrations for the corresponding antigen (CD4, CD45). After incubation, the non-adherent CEM cells were harvested. The absence of contaminating HEK 293 cells was controlled by FACS analysis using cell-surface labelling of CD45 antigen on harvested cells (in each experiment more than 99% cells were CEM). The percentage of apoptotic cells was measured by flow cytometry analysis using YOPRO-1.

### ACKNOWLEDGMENTS

We thank Patrice Lassus (IGMM, Montpellier) for helpful discussions. We also thank Pierre Corbeau and Leigh Dawson (CRBM, Montpellier) for critical reading of the manuscript. This work was supported by Institutional funds from the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), a grant (to C. D.) from the Agence Nationale de Recherches sur le SIDA (ANRS); C. G. is fellow of the ANRS.

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