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Immunology

Stimulation of a human T-cell clone with anti-CD3 or tumor necrosis factor induces NF-κB translocation but not human immunodeficiency virus 1 enhancer-dependent transcription

(long terminal repeat/transcription factors/lymphokines/T-cell clone/antigen recognition)

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ABSTRACT **The expression of transiently transfected expression vectors under the control of the long terminal repeat (LTR) of the human immunodeficiency virus (HIV) or its enhancer sequence and the translocation of the HIV enhancer-binding protein NF-κB were analyzed in two human T-cell clones stimulated through their T-cell receptor complex or by tumor necrosis factor or phorbol 12-myristate 13-acetate. We found a dissociation of NF-κB translocation from transactivation of either the HIV LTR or the HIV enhancer. Interleukin 2 induced proliferation but not NF-κB translocation or LTR transactivation. Phorbol ester or specific antigen recognition induced HIV LTR transactivation, whereas stimulation with tumor necrosis factor or antibody to CD3 did not. The two latter signals were nevertheless able to induce NF-κB translocation with a pattern in the band-shift assay indistinguishable from that observed using phorbol ester. Our finding that induction of NF-κB by tumor necrosis factor or antibody to CD3 is not sufficient to induce HIV enhancer-dependent transcription in cloned T cells contrasts with results obtained in most lymphoblastoid T-cell lines and indicates that normal T lymphocytes differ from tumoral T cells in terms of requirements for HIV LTR activation. Furthermore, our results suggest that events linked to T-cell activation, in addition to NF-κB translocation *per se*, induce functional interactions of the NF-κB complex with the HIV enhancer.**

Among circulating leukocytes (peripheral blood lymphocytes) of human immunodeficiency virus (HIV)-seropositive patients, CD4-positive T lymphocytes represent the main cell type infected (1). Such cells, when cultured *in vitro*, show very little, if any, HIV replication. Virus production is induced in peripheral blood lymphocyte cultures from most patients by stimulation with mitogens, such as phytohemagglutinin or phorbol 12-myristate 13-acetate (PMA) (2). This indicates that transcriptional activation of HIV provirus from a quiescent state in resting, circulating lymphocytes is under the control of cellular genes induced during T-cell activation, as has been discussed (3). Analysis of molecular events linking T-cell activation to HIV provirus activation is essential for our understanding of the pathogenesis of AIDS. Most of our knowledge in this field comes from the study of HIV long terminal repeat (LTR) activity in tumoral lymphoblastoid T-cell lines. In such cell systems, it has been shown that the HIV LTR is the main region regulating transcription. It contains a 10-base-pair direct repeat enhancer element that can bind and respond to NF-κB-like transcription factor(s) (4) translocated from the cytoplasm upon stimulation with PMA or mitogens (5). Two natural cytokines, tumor necrosis factor (TNF) and lymphotoxin (LT), have also been shown to induce both HIV replication and HIV LTR transactivation through translocation into the nucleus of NF-κB-like protein(s) that can bind to the HIV enhancer (6–8). In addition, mutation of the 10-base-pair repeat sequence that binds NF-κB abolishes mitogen-induced HIV LTR transactivation in lymphoblastoid T-cell lines (5).

It is important to understand which physiological signals induce HIV transcription in normal T lymphocytes. Indeed, mitogens are artificial reagents and TNF may not be able to activate resting T lymphocytes that do not express detectable TNF receptors (9). A physiological event that can activate CD4-positive T lymphocytes from their normal resting state is antigen recognition (10). Our approach has been to use as an experimental system, CD4-positive T-lymphocyte cell clones maintained in interleukin 2 (IL-2)-rich medium and transfected with HIV LTR expression vectors. The advantages of this cell system are manifold: (*i*) cloned T cells are not tumoral, (*ii*) they express functional membrane receptors, (*iii*) they respond to and produce lymphokines such as IL-2, TNF, LT, and interferon-γ (IFN-γ), and (*iv*) they can be directly activated by specific antigen presentation. In this system, we found that the requirements for HIV LTR transactivation are different from those reported in tumoral T-cell lines.

MATERIAL AND METHODS

Cells. Clone D106 (a kind gift from M. Agrapart and J. J. Ballet, Hôpital St. Louis, Paris) is a human CD4-positive, CD3-positive, CD8-negative T-cell clone that specifically responds to tetanus toxoid when cocultured with autologous macrophages (11). Clone SPB21 is a CD4-positive, CD3-positive, CD8-negative human T-cell clone that was selected through specific proliferation triggered by anti-T-cell receptor (TCR)–CD3 monoclonal antibody (mAb) 3D6 (12). After thawing, SPB21 cells and irradiated allogenic peripheral blood leukocytes were stimulated with phytohemagglutinin (0.1 μg/ml, Wellcome) and cocultured for 3 days as described (12). On day 3, and every 3 days thereafter, recombinant (r) IL-2 (Cetus) at 20 ng/ml was added to the cultures. The human lymphoblastoid cell line J-Jhan, a subclone of the Jurkat cell line, was cultured and transfected as described (8). 70Z/3 is a pre-B-cell line (13).

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Abbreviations: CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus; HEBP, HIV enhancer binding protein; IFN-γ, interferon-γ; IL-2, interleukin 2; LT, lymphotoxin; LTR, long terminal repeat; Luc, luciferase; PMA, phorbol 12-myristate 13-acetate; TK, thymidine kinase; TNF, tumor necrosis factor; r, recombinant; TCR, T-cell receptor; CRE, cAMP-responsive element; mAb, monoclonal antibody.

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Lymphokine Assays. Supernatants from cell cultures were titrated for IFN-γ and TNF/LT production with standard bioassays as described (14).

Culture Medium, Lymphokines, and Antibodies. Yssel's medium, an Iscove's modified Dulbecco's medium (GIBCO/BRL) (15), was supplemented with 1% heat-inactivated pooled human AB serum. PMA was purchased from Sigma. rIL-2 was from Cetus. Human rTNF and neutralizing rabbit antisera to TNF and LT were a gift from W. Fiers (Rijksuniversitat, Ghent, Belgium). Purified anti-TNF mAb TE115 was a gift from D. Lando (Roussel-UCLAF). mAb SPV T3b, an IgG2a directed against CD3 molecule (16), was used as crude ascites fluid.

Plasmids. We constructed a series of Luciferase (Luc) expression vectors (17) under the control of various HIV LTR fragments or of the weak truncated herpes simplex thymidine kinase (TK) promoter (positions −105 to +51). The HIV LTR fragment [*Xho* I (−640)–*Hin*dIII (+78)] containing the U3 and R regions was cloned into the pC-Luc plasmid, generating pLTRX-Luc. A shorter LTR fragment [*Sca* I(−142)–*Hin*dIII(+78)] was cloned in pC-Luc, yielding plasmid pLTRS-Luc. The p3enhTK-Luc vector was constructed by cloning the 270-base-pair *Bgl* II(Klenow)–*Sal* I fragment of plasmid pHI-03TK-CAT, as described (8), into the *Hin*dIII(Klenow)–*Sal* I sites of the pC-Luc polylinker (17). This vector contains three copies of the HIV-enhancer synthetic oligonucleotide described below in the band-shift assay. Various *tat* expression vectors were used. Vector pLTRA-tat contained the *Ava* I(−158)–*Hin*dIII(+78) HIV LTR fragment upstream of tat cDNA with the simian virus 40 poly(A) region. The pCMV-tat vector contained a tat cDNA with the simian virus 40 poly(A) region inserted downstream of the human cytomegalovirus immediate early enhancer/promoter (17). Finally the pLTRX-CAT used in experiments shown in Fig. 1 carries the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the *Xho* I–*Hin*dIII LTR fragment described above.

Cell Transfection. D106 cells (2×10^7 cells) were transfected with 5–10 μg of plasmid(s) by using a modified DEAE-dextran technique (8). Transfected cells were dispatched and layered on autologous irradiated monocytes used as antigen-presenting cells. Tetanus toxoid (purified tetanus toxoid, BioMerieux, Charbonnier les Bains, France) was used at 10 μg/ml. Ten days after thawing SPB21 cells (5×10^7 to 1×10^8 cells) were transfected with 20–30 μg of plasmid(s) by electroporation in RPMI 1640 medium (GIBCO/BRL) with 5% (vol/vol) human AB serum in the absence of rIL-2. A single pulse at 680 V/cm and 1340 μF was done. After the electric shock, cells transfected with the same plasmid(s) were pooled, washed, and incubated for 8 hr, before stimulation by anti-CD3 (ascites fluid dilution, 1:500), rTNF (500 units/ml), PMA (10 ng/ml), or rIL-2 (20 ng/ml).

Luc and CAT Assays. These were performed as described (8, 17) 18–20 hr after cell stimulation. Luc index represents the Luc activity relative to the cell number and was calculated using the following formula: (Luc cpm − background cpm)/(number of living cells $\times 10^{-6}$).

Band-Shift Assay. For preparation of nuclear proteins, SPB21 cells were washed and deprived of rIL-2 for 16–18 hr before being stimulated as described above. 70Z/3 pre-B cells were stimulated by PMA (25 ng/ml), thus providing bona fide NF-κB-positive control (13). Nuclear extracts from control and stimulated cells were then prepared and tested as described (8, 18). Double-stranded oligonucleotides corresponding to either wild-type or mutated enhancer repeat (NF-κB-like binding site) of HIV LTR enhancer (a gift from C. Auffray and A. Delaroche, Institut d'Embriologie, Nogent sur Marne, France) or to the immunoglobulin κ chain NF-κB binding site (gift from A. Israël, Institut Pasteur, Paris) were labeled at the 5′ end. Specific binding was controlled by competition with a 50-fold excess of either unlabeled HIV enhancer or unlabeled irrelevant oligonucleotide [corresponding to the cAMP-responsive element (CRE) of the somatostatin gene (19), a gift from M. Yaniv (Institut Pasteur, Paris)].

Sequences of the oligonucleotides used are as follows: HIV enhancer, 5′-AGCTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGA-3′; HIV enhancer mutant (mutated sites are underlined), 5′-ACTCACTTTCCGCTGCTCACTTTCC-3′; immunoglobulin κ-chain enhancer, 5′-GACAGAGGGGACTTTCCGAGAGG-3′; somatostatin CRE, 5′-GTTGGCTGACGTCATCAAGCTA-3′.

RESULTS

Cells of the D106 clone were transiently transfected with an HIV-LTR-CAT construct and specifically stimulated with tetanus toxoid. An increase in CAT activity was observed in antigen-stimulated cultures, and a further increase was found in stimulated cells cotransfected with the HIV-LTR-tat vector, as can be seen in the representative experiment shown in Fig. 1. It can be seen in Fig. 2 that the basal level of LTR activity (pLTRS-Luc vector) was remarkably low in T-cell clone SPB21 and was poorly enhanced by cotransfection of a vector permitting tat protein expression under the control of the autologous LTR. In contrast, cotransfection of *tat* driven by the strong cytomegalovirus immediate early promoter (20) led to transactivation of the HIV-LTR-Luc vector. We tested the effects of PMA and rTNF on the activity of LTR-Luc constructs electroporated with or without an LTR-tat expression vector. Fig. 2 shows that PMA stimulated activity of the HIV LTR, an effect further increased by cotransfection of an LTR-tat vector. In contrast, rTNF was unable to induce HIV LTR transactivation, even when cotransfected with pLTR-tat, which would be expected to synergize in the system. In parallel experiments, the same

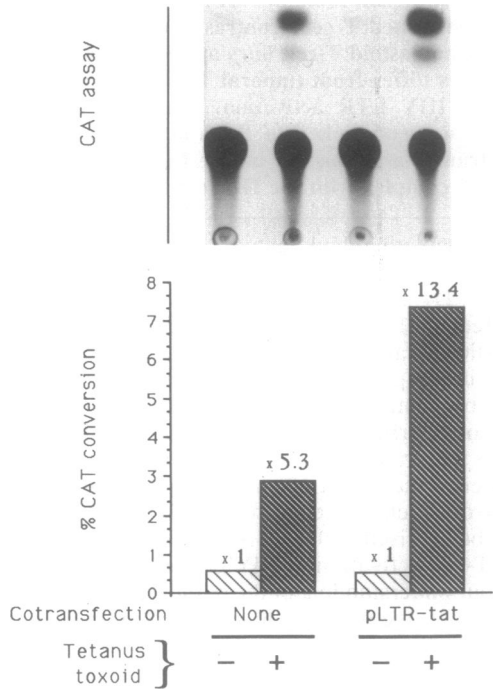


FIG. 1. Antigen presentation induces HIV LTR transactivation in the D106 T-cell clone. The pLTRX-CAT vector was transfected alone or cotransfected with a pLTR-tat expression vector into the D106 T-cell clone by using DEAE-dextran (Sigma). After transfection, T cells were cocultured with adherent autologous monocytes in the presence (+) or absence (−) of tetanus toxoid (10 μg/ml). CAT activity was measured in cell lysates 24 hr after stimulation. Figures at the top of columns represent fold amplification relative to CAT conversion in the absence of antigen stimulation.

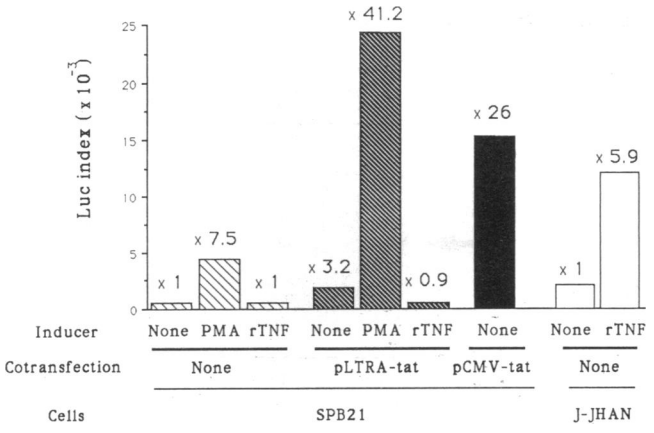


FIG. 2. HIV LTR activity in the SPB21 T-cell clone after electroporation of the pLTRS-Luc vector. Cells were stimulated by PMA or rTNF without (■) or with (■) cotransfection of pLTRA-tat. The effect of cotransfection of pCMV-tat is also shown (■). The same preparation of rTNF (500 units/ml) was used to enhance expression of the pLTRS-Luc vector transfected alone in the J-Jhan T-lymphoblastoid cell line (□). Figures at the top of columns represent fold amplification relative to Luc expression in unstimulated cells transfected with pLTRS-Luc alone.

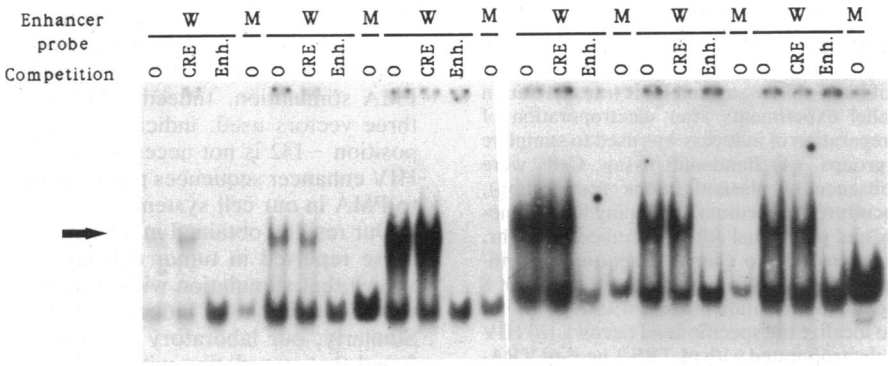
preparation of rTNF was able to induce LTR transactivation in the human lymphoblastoid T-cell line J-Jhan, as reported (8). No transactivation of the HIV LTR was observed in SPB21 clone stimulated by anti-CD3 antibody or rIL-2. Stimulation with antibody to CD3 induced T-cell activation, as measured by cell proliferation and secretion of TNF, LT, and IFN-γ, but did not stimulate the activity of the LTR (see Figs. 3 and 4). Addition of rTNF or rIL-2 was similarly ineffective (mean of amplification, 0.9 ranging from 0.7- to 2-fold; data not shown). In contrast, PMA induced both lymphokine secretion and activation of the HIV LTR.

The effect of the above described signals on HIV enhancer binding protein (HEBP) induction in the SPB21 clone, compared to their ability to stimulate cell proliferation and lymphokine production, is shown in Fig. 3. Addition of rIL-2 induced intense cell proliferation, but very little (not detectable in most experiments) induction of HEBP, TNF, LT, or IFN-γ. In contrast, stimulation with PMA, rTNF, or anti-CD3 mAb always resulted in a clear induction of a specific HEBP of comparable intensity whatever the inducer used. It should be noted that CD3 stimulation induced modest, but clearly detectable proliferation (proliferation index range from 3 to 5) and also secretion of TNF/LT and IFN-γ. However, complete neutralization of TNF and LT by specific antibodies during stimulation by anti-CD3 did not significantly decrease HEBP induction.

Fig. 4 shows a representative experiment in which HEBP induction and transactivation of either HIV-LTR or enhancer TK-Luc vectors were performed in parallel, using the same culture conditions. Cells used for band-shift assay were thus electroporated without plasmid. We had observed that T-cell clones tended to release TNF and LT transiently in the hours after electroporation. To avoid NF-κB induction by TNF/LT before stimulation, we performed the experiment shown in Fig. 4 in the continuous presence of excess neutralizing anti-TNF and anti-LT antibodies before washing and stimulation. Under these conditions, no TNF/LT activity was detectable at any time before stimulation. Supernatants of unstimulated cultures remained TNF/LT-negative after removal of the neutralizing antibodies. Stimulation of the culture at that stage induced TNF and LT secretion. It can be seen (Fig. 4*A*) that comparable levels of a specific binding on the HIV-enhancer oligonucleotide and lymphokine secretion were obtained with the three inducers (anti-CD3, rTNF, and PMA). It was again observed that PMA, but not anti-CD3 antibody or rTNF, induced transactivation of the two vectors used (Fig. 4*B*). Fig. 5 shows that the migration patterns induced by the anti-CD3 antibody, rTNF, and PMA were indistinguishable from one another and from that of bona fide NF-κB protein induced in 70Z/3 pre-B cells by PMA (13).

DISCUSSION

The present report demonstrates that antigen recognition by a human T-cell clone results in transactivation of the HIV-LTR vectors transfected in such nontumoral cells. This was observed in cultures of a CD4-positive tetanus-toxoid-specific T-cell clone



Proliferation index	1	66	2	2	3.5	11
TNF/LT (U/ml)	<2	<2	128	ND	48	<2
IFN-γ (U/ml)	<3	<3	18	9	36	24

FIG. 3. Identification of specific nuclear factors binding to the HIV enhancer in SPB21 cells incubated for 16 hr with PMA, rTNF, rIL-2, or anti-CD3 (α-CD3). Polyclonal antibodies to TNF (α-TNF) and LT (α-LT) were added to the cell culture before stimulation with α-CD3. Band-shift assay was performed using either wild-type (W) or mutated (M) labeled HIV enhancer probe. The specific band is shown by an arrow. Culture supernatants were collected 16 hr after stimulation for analysis of TNF/LT (ND, TNF/LT activity was not determined when rTNF was used as inducer) and IFN-γ. Proliferation index is the ratio of [^{3}H]thymidine incorporation by induced cells relative to unstimulated cells. U/ml, unit(s)/ml; Enh., enhancer.

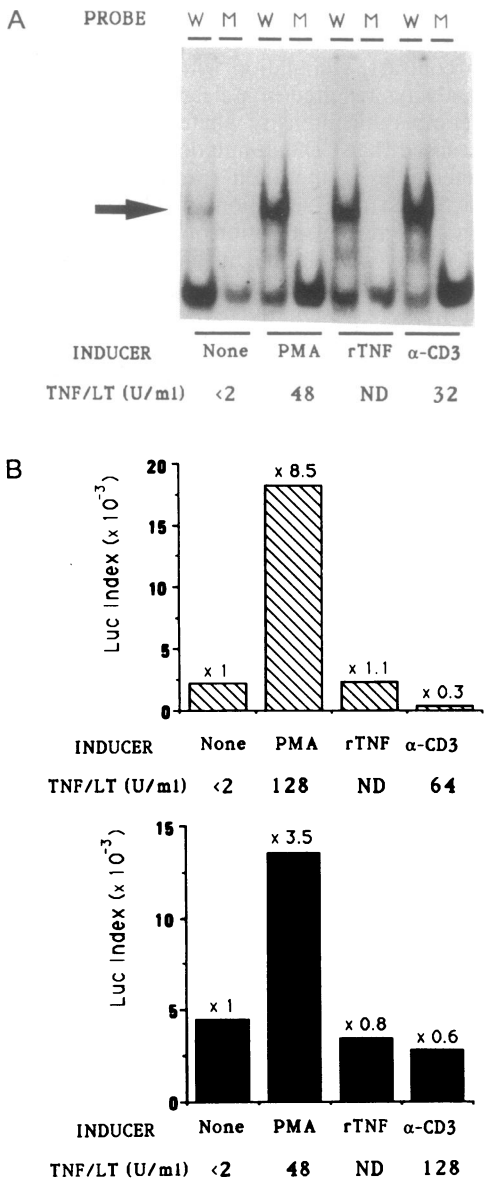


FIG. 4. Comparison of band-shift assay and LTR transactivation results obtained in parallel experiments after electroporation of SPB21 cells. The same preparation of inducers was used to stimulate the three experimental groups. (*A*) Band-shift assay. Cells were electroporated in the absence of plasmid (mock transfection), pooled, washed, and subcultured in medium containing both monoclonal anti-TNF antibody and polyclonal anti-LT antibody for 8 hr. Cells were then washed extensively to eliminate neutralizing antibodies to TNF and LT and stimulated for an additional period of 8 hr. Labeled HIV enhancer oligonucleotide probes, wild-type (W) or mutated (M), were used to localize the specific band (arrow). (*B*) HIV LTR activity. Cells were electroporated with pLTRS-Luc + pLTRA-tat (▨) or p3enhTK-Luc (■). Culture conditions and stimulation as described in *A* except that cell lysates for Luc assay were prepared 16 hr after cell stimulation. Figures at the top of columns represent fold amplification relative to unstimulated cells. U/ml, unit(s)/ml; α-CD3, anti-CD3.

stimulated with antigen in the presence of autologous adherent monocytes. This observation, obtained with a pure cell population directly triggered by tetanus toxoid antigen through the TCR–CD3 complex, confirms and extends previously reported data (21, 22). Indeed HIV replication has been found to be enhanced in tetanus-toxoid-stimulated peripheral blood lymphocytes infected with HIV (21), and HIV-LTR vectors have been shown to be induced in primary T-cell blasts obtained after stimulation with purified protein derivative (22).

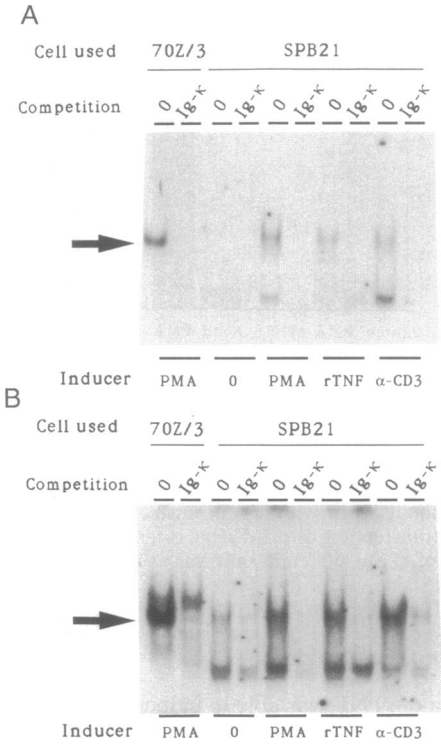


FIG. 5. Band-shift assay using oligonucleotide probes with the sequences of either the immunoglobulin κ (Ig-κ) chain (*A*) or the HIV enhancer (*B*) in nuclear extracts from the 70Z/3 cell line stimulated with PMA or SPB21 T cells stimulated with PMA, rTNF, or anti-CD3 (α-CD3).

Three types of HIV-LTR-Luc vectors were transfected into a CD4-positive IL-2-dependent T-cell clone (SPB21) that can be activated by soluble anti-CD3 antibody (23) to secrete TNF, LT, and IFN-γ. In this cell system, such stimulation did not result in detectable activation of the LTR using various transfected vectors, despite evidence of cell activation. rIL-2 induced intense cell proliferation but no LTR transactivation. Addition of rTNF did not modify the activity of the vectors used. These negative results were not due to an intrinsic inability of the T-cell clone environment to permit transactivation of the HIV LTR, since it clearly responded to PMA stimulation. Indeed, PMA induced activation of the three vectors used, indicating that the region upstream of position −142 is not necessary for PMA induction and that HIV enhancer sequences played a major role in the response to PMA in our cell system.

Our results, obtained in a normal T-cell clone, differ from those reported in tumor cell lines. In Jurkat cells, it was shown that stimulation with immobilized OKT3 antibody to CD3 is sufficient to induce HIV LTR transactivation (24). Similarly, our laboratory (8) and other groups (6, 7) have found that stimulation with rTNF alone is able to stimulate HIV transcription in tumor T-cell lines.

CD3 stimulation and rTNF induced HEBP in SPB21 cells in a manner similar to PMA. HEBP induction by anti-CD3 antibody was not a consequence of endogenous TNF/LT secretion, since complete neutralization of TNF and LT by specific antisera did not significantly diminish CD3-triggered HEBP induction. As neutralization of TNF in cell cultures is highly efficient, this indicates that CD3 stimulation induces HEBP through a pathway at least partially independent of autocrine secretion of TNF and LT. It should be noted that rIL-2 induced intense proliferation but showed a very modest and variable effect on HEBP induction in our T-cell clone. Thus T-cell proliferation *per se* can be dissociated from HEBP induction and HIV LTR transactivation.

It remains to be understood why CD3 simulation and rTNF induced HEBP but not LTR transactivation, whereas PMA did both. To explain this surprising difference, two hypotheses can be envisaged. One is that PMA, known to induce a wide repertoire of transcription factors (25), could activate, apart from protein(s) efficient in HIV enhancer binding and induction, factors that would interact functionally with other sequences in the LTR. This is unlikely, however, because similar results were obtained when a TK-Luc vector driven by the synthetic oligonucleotide sequence of the HIV enhancer was used instead of the HIV-LTR-Luc constructions. The second possibility would be that the protein(s) induced by PMA or the two other inducers used bind to the HIV enhancer but differ qualitatively. Indeed, the HIV enhancer is able to bind purified NF-κB protein (26) but also protein factors with a different molecular weight (27, 28). In our band-shift assays, however, the protein factor(s) induced by anti-CD3 antibody or rTNF behaved indistinguishably from HEBP induced by PMA or bona fide NF-κB obtained from PMA-induced 70Z/3 cells (13, 26). Such properties are compatible with the interpretation that the NF-κB protein complex (29) was the enhancer-binding factor detected in our cell system, whatever the inducer used. If this interpretation is correct, we are bound to conclude that induction of NF-κB binding activity in the nucleus is not a sufficient condition for the HIV enhancer to be transactivated in nontumoral T lymphocytes, such as T-cell clones. In normal T cells, events linked to cell activation appear to be necessary, in addition to NF-κB translocation, to induce functional interactions of the NF-κB protein complex with the HIV enhancer. This could occur through either induction of another factor undetectable in band-shift assays, which would act in combination with NF-κB or, more probably, through post-translational modifications of one or more protein components of the NF-κB complex (29). Such dissociation between DNA–protein interaction and functional induction is not unprecedented, as shown with the c-*fos* promoter (30).

We observed that NF-κB induction, presumably resulting from TCR–CD3-induced protein kinase C activation, was not sufficient to induce LTR transactivation whereas antigen recognition, known to involve costimulatory signals, resulted in HIV LTR induction. It appears that several activation signals, such as those provided by antigen-presenting cells, must synergize to attain both full T-cell activation and HIV LTR transactivation. In IL-2-dependent cloned T lymphocytes, such factor(s) would be inducible by PMA and antigen recognition, but not by TNF and soluble antibody to CD3. Stimulation by appropriate ligands of single transmembrane receptors, such as the CD3 complex or the TNF receptor is sufficient for induction of NF-κB translocation but not for induction of HIV LTR-dependent transcription. These observations limit the hypothesis that exogenous or autocrine secretion of TNF alone has a major role *per se* in HIV provirus activation, although it may amplify it. Specific antigen recognition through the TCR–CD3 complex, in association with other membrane molecules and secondary secretion of cytokines, is the physiological signaling system inducing full activation of T cells from their basal resting state and is likely to be the main trigger of HIV transcription in infected human T lymphocytes.

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