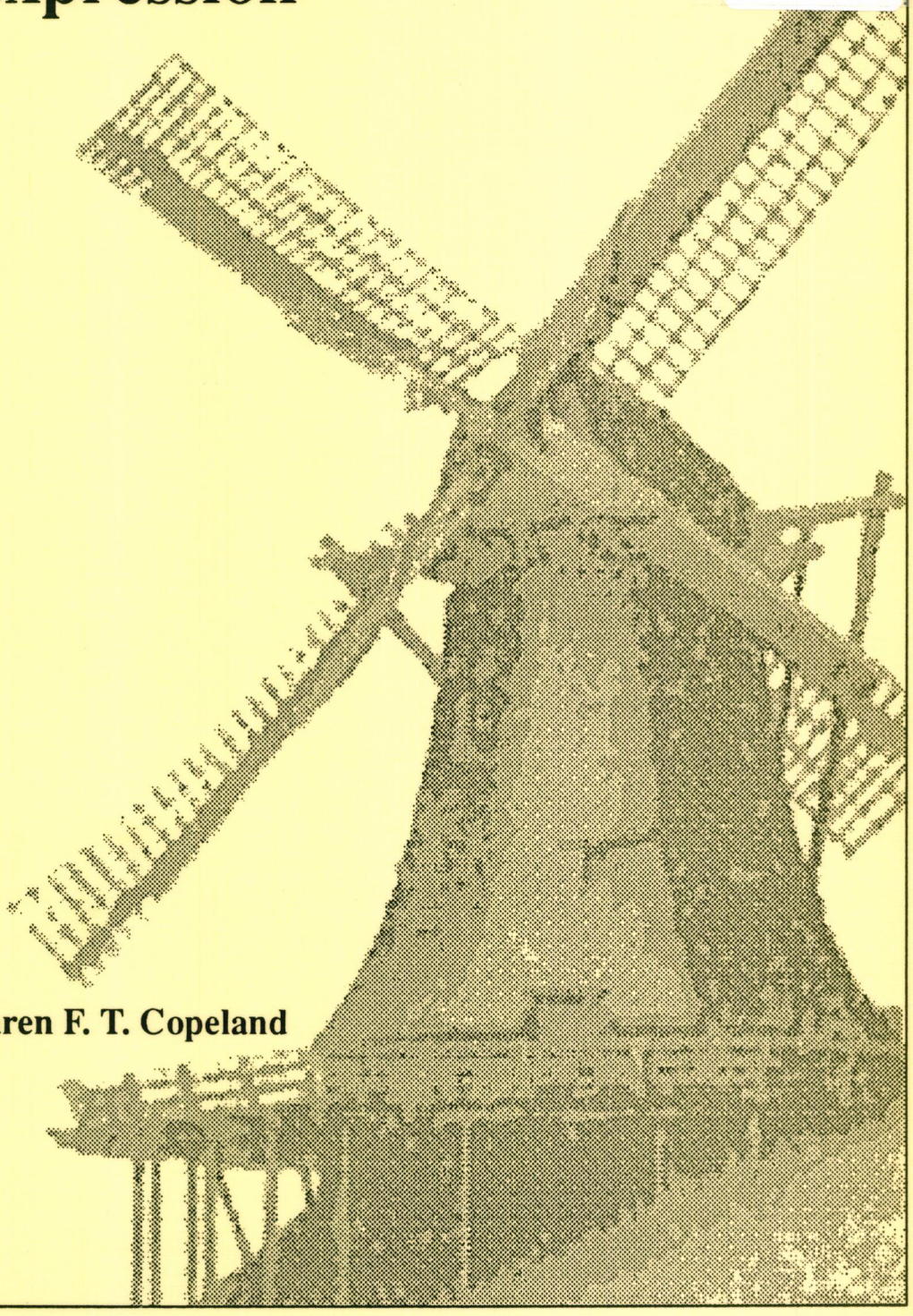
The Role of T-cell Activation in Human Retroviral Expression



Karen F. T. Copeland

THE ROLE OF T-CELL ACTIVATION IN HUMAN RETROVIRAL EXPRESSION

An investigation of the influence of T-cell signal transduction pathways on human retroviral expression with particular reference to HTLV-1

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Stellingen of the thesis of Karen Copeland
"The role of T-cell activation in human retroviral expression"

1 While HIV-1 expression is upregulated by T-cell activation pathways, these same signals contribute to the inhibition of HTLV-1 expression. T-cell activation may thus support viral quiescence and the prolonged asymptomatic period of ATL.
(this thesis)

2 Components of the calcium pathway stimulated by ionomycin inhibit the expression of HTLV-1 while enhancing the expression of HIV-1.
(this thesis)

3 The protection against apoptosis afforded by HTLV-1 *tax* may provide a mechanism to support the expansion of the population of infected cells.
(this thesis)

4 *Tax*-mediated inhibition of apoptosis is dependent on the availability of PKC.
(this thesis)

5 The recovery of ionomycin inhibition of HTLV-1 expression by cyclosporin A may reveal a new activation pathway.
(this thesis)

6 Antigen-cytokine fusion proteins provide a potential improvement over current approaches to tumour vaccines.
M.-H. Tao and R. Levy Nature 1993 362: 755-758.

7 The maintenance of T-cell memory may be dependent on continuous restimulation of $CD45RO^+$ T-cells to enhance bcl-2 expression (and prevent apoptosis).
A.N. Akkar et al. 1993 J. Exp. Med. 178: 427-438.

8 The interaction between NFATp and Fos and Jun provides a mechanism for combinatorial regulation of IL-2 gene transcription which integrates the Ca^{2+}- and PKC-dependent pathways of T-cell activation.
J. Jain et al. Nature 365: 352-355.

9 Life is an endless series of experiments.
(Mohandas K. Gandhi)

10 The "dagje uit" is the working Dutchman's way of "stopping to smell the tulips".

For my mother

CONTENTS Page

ABBREVIATIONS

AIDS	-	acquired immunodeficiency syndrome
ATL	-	adult T-cell leukemia
BAPTA	-	1,2 bis(2-aminophenoxy) ethane N,N,N',N' tetraacetic acid
CAT	-	chloramphenicol-acetyl transferase
CRE	-	cyclic AMP responsive element
CsA	-	cyclosporin A
DAG	-	diacylglycerol
EGTA	-	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N' tetraacetic acid
FCS	-	fetal calf serum
FDG	-	fluorescein-di-β-D-galactopyranoside
G418	-	gentamicin sulphate
HBSS	-	Hank's balanced saline solution
HIV	-	human immunodeficiency virus
HLA	-	human leukocyte antigen
HTLV	-	human T-cell leukemia virus
IL-2	-	interleukin 2
IL-2R	-	interleukin 2 receptor
IP3	-	inositol triphosphate
kDa	-	kilodalton
LacZ	-	β-galactosidase
LTR	-	long terminal repeat
mAb	-	monoclonal antibody
MHC	-	major histocompatibility complex
MUG	-	4-methylumbelliferyl-β-D-galactopyranoside
NF-AT	-	nuclear factor of activated T-cells
NFκB	-	nuclear factor kappa B
NRE	-	negative regulatory element
PBMC	-	peripheral blood mononuclear cell
PBL	-	peripheral blood lymphocyte
PBS	-	phosphate buffered saline
PHA	-	phytohaemagglutinin
PKC	-	protein kinase C
RE	-	responsive element
RRE	-	rev responsive element
RxRE	-	rex responsive element
SIV	-	simian immunodeficiency virus
TCR	-	T-cell receptor
TAR	-	transactivation response element
TNFα	-	tumor necrosis factor alpha
TPA	-	12-O-tetradecanoylphorbol-13-acetate
TRE	-	tax responsive element
X-Gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

I HUMAN RETROVIRUSES

Two different subfamilies of retroviruses associated with human disease have been identified. Their isolation has been facilitated by the discovery of T-cell growth factor (TCGF) and the subsequent cloning of cytokines important for T-cell proliferation such as interleukin-2 (IL-2) (99, 205, 209, 329). In 1979, van der Loo and colleagues (303) reported C-type retrovirus particles and reverse transcriptase activity in Langerhans cells and related cells of skin and lymph nodes of patients with Sezary's syndrome and mycosis fungoides. Similar virus activity was later detected and virus isolated from lymphocytes of patients with Sezary's syndrome and cutaneous T-cell lymphoma (236, 237). This virus, human T-cell leukemia virus type-1 (HTLV-1), was found to be associated with adult T-cell leukemia /lymphoma (ATL), a malignancy of mature helper T (T_H) lymphocytes (105, 106, 236, 323). A second retrovirus was later isolated from T-lymphocytes of a patient with hairy T-cell leukemia (133) and was named HTLV-2. In addition to ATL, HTLV-1 has more recently been associated with other diseases, including HTLV-1-associated myelopathy (229), tropical spastic paraparesis (83) and HTLV-1-associated arthropathy (220). Fewer than 1% of HTLV-1 seropositive individuals have been reported to develop ATL, and only after a clinical latency period spanning decades of subclinical infection (105).

The initial isolation of HTLV-1 was facilitated by the discovery of IL-2 which made possible the long-term culturing of human T-lymphocytes (205, 209). The isolation of human immunodeficiency virus type-1 (HIV-1) proved to be dependent upon the use of mitogenic agents which induce T-cell activation and virus expression (99, 329). HIV-1 is a lentivirus which causes a selective loss of CD4+ T-cells and has been identified as the primary etiologic agent of acquired immune deficiency syndrome (AIDS) (13). A second virus in this family, HIV-2, is a causative agent of AIDS in Western Africa (38, 134) and

shares approximately 40% nucleic acid similarity with HIV-1 (38, 41, 95, 109). Sequence similarities of HIV-2 and SIVsmm (simian immunodeficiency virus derived from sooty mangabeys), combined with the cross-reactivity of antibodies to both viruses, suggest that HIV-2 and SIV may have a common origin or may have derived from one another (62, 162, 198, 272).

These retroviruses share the common characteristic of chronic life long infection. During a long and variable asymptomatic period, virus expression in peripheral blood is minimal and overt clinical disease is not manifested. Substantial evidence exists suggesting that human retroviruses require activated T-cells to initiate permanent infection (89, 285, 328). Furthermore, accumulating data exist to suggest that transcriptional signals used by T-cells to regulate T-cell function are also recognized by these retroviruses to regulate virus production. In effect, human retroviruses require an active immune system to establish infection and subsequently propagate. The way in which these viruses respond to the intracellular signals produced in T_H-lymphocytes, following immune response to common antigens encountered daily by the host, may lead to two extremely different outcomes. Depending on whether infection is with HTLV or HIV, the long-term effect on the host may be either dysregulated T_H-cell proliferation leading to ATL or T_H-cell loss leading to systemic immune suppression and AIDS.

II THE CELLULAR IMMUNE RESPONSE

T-cell Activation and Cell Proliferation

T-lymphocytes develop to respond to specific non-self antigens upon primary exposure to new antigens in the course of immune system development. Thereafter memory lymphocytes exist in a quiescent state (G_0) for most of their lifespan. Many genes required during cell division and function are transcriptionally silent during this period.

Following subsequent exposure to an antigen, recognized by a T-cell receptor (TCR) specific for the particular antigen, the lymphocyte enters the G_1 phase of the cell cycle with subsequent expression of genes employed in T-cell activation and function. In the case of T_H-lymphocytes, binding of antigen to its specific T-cell receptor in the context of the appropriate MHC class II molecules, together with CD4 binding and the appropriate secondary cytokine signals provided by antigen presenting cells, results in T_H-cell proliferation and ensuing immune function. Lymphocyte proliferation is mediated by specific intracellular pathways which become activated following the interaction of antigen with the TCR at the cell surface (Figure 1). Triggering of the phosphatidyl inositol pathway by this interaction results in the cascade of second messengers which are essential for the expression of genes required for proliferation and subsequent immune function. Upon binding of antigen to the TCR, the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) are generated by the hydrolysis of membrane bound phosphatidyl inositol 4,5-bisphosphate by phospholipase C (39). DAG directly activates protein kinase C (PKC) resulting in the translocation of the phosphoprotein to the cell membrane (17, 221). The production of IP3 triggers a dramatic bimodal elevation of free calcium within the cell (39, 80). The release of calcium from intracellular stores produces a transient surge which is followed by a second more prolonged surge as calcium enters the cell from the extracellular environment (82). Activation of these signalling pathways via the TCR, combined with a secondary signal which can be provided by IL-1 (52), result in the expression of IL-2 and IL2 receptor (IL-2R) and subsequent cellular proliferation. Signalling of the T-cell via the TCR is required for entry into the G_1 phase of the cell cycle. Progression from G_1 is precisely determined by the density of IL-2R and the absolute number of IL-2/IL-2R interactions (26). Essential to the expression of IL-2 and IL-2R is the activity or upregulation of immediately expressed cellular factors required for transcription, including NF-AT, NFκB and cellular proto-oncogene products (Figure 1).

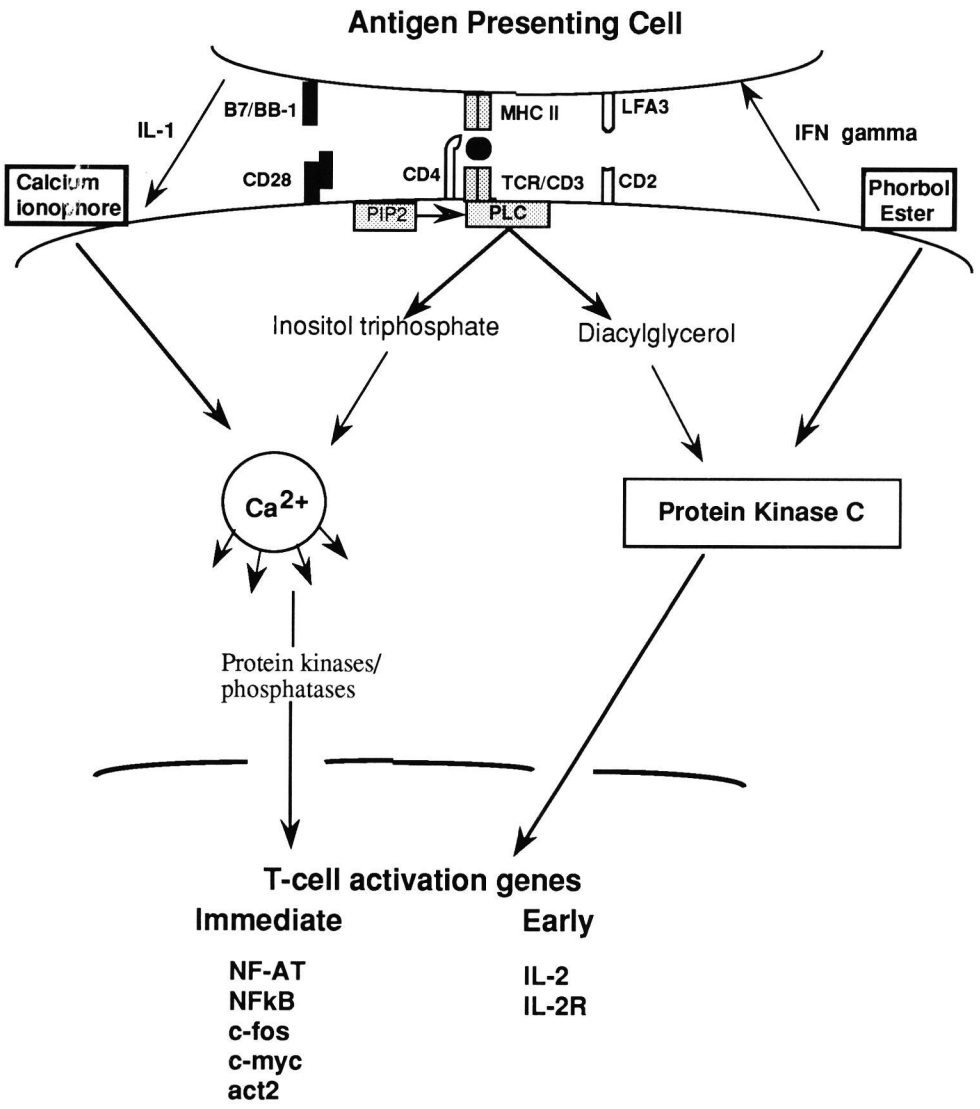

Figure 1. *Biochemical pathways of T-cell activation in the T helper-lymphocyte.*
The stimulation of the phosphatidyl inositol pathway by binding of antigen to the TCR/CD3 complex triggers the activation of transcription factors required for early gene activation leading to cellular proliferation. PIP2, phosphatidylinositol bisphosphate; PLC, phospholipase C; LFA, lymphocyte function antigen.

T Helper Lymphocyte Function

The way in which a T_H-cell is able to respond to immune signals is restricted by phenotype. T_H1-cells produce IL-2 and IFNγ which promote cell-mediated effector responses, such as the activation of macrophages for the destruction of intracellular parasites. The T_H2-cell is characterized by the production of IL-4, IL-5, IL-6 and IL-10 which influence B-cell development and humoral responses. The production of T_H1-cells is favoured during viral infection, presumably by the production of high levels of IFNγ and the absence of IL-4 (42, 78, 183). The opposite is true for T_H2-cell production which is favoured by allergen-induced IL-4 production and an absence of IFNγ (161, 183).

T-cell specificity is determined during ontogeny in the thymus. The diversity of expressed TCRs allows for a large T-cell repertoire which must undergo both positive and negative selection. Positive selection provides for the maturation of thymocytes bearing TCRs which recognize self MHC molecules (18). Upon maturity, this population of lymphocytes can respond to normal immunological signals (activation). Recognition of self-reactive molecules is prevented by negative selection mediated by clonal inactivation (anergy) and clonal deletion (apoptosis). Mature T-cells are resistant to pathways which induce apoptosis and can proliferate in response to normal TCR stimulation. Under certain conditions however, stimulation of a mature T-cell via the TCR may result in aberrant induction resulting in anergy or apoptosis (216).

III HTLV INFECTION

Extracellular Viral Effects

The cell surface receptor recognized by HTLV-1 remains unidentified. Sommerfelt et al. (282) demonstrated, using vesicular stomatitis virus pseudotypes bearing HTLV-1 glycoproteins, that somatic cell hybrids with human chromosome 17q were susceptible to

infection by HTLV-1. *In vitro*, HTLV-1 has been shown to infect several different cell types, including CD4+ lymphocytes, although only human T-lymphocytes have been demonstrated to become transformed (101, 248). This specificity correlates with the observation that ATL cells are always of the helper phenotype (101). Integration of the provirus occurs randomly with one cell selected during *in vitro* culture. *In vivo*, primary tumor cells are monoclonal with respect to the integration site of the provirus (318, 323, 327). The *in vitro* observation that only T-lymphocytes may be transformed by HTLV-1 may suggest a T-cell specific life cycle event as key to providing the appropriate conditions for transformation.

HTLV-1, even when inactivated, has been demonstrated to serve as a mitogenic signal capable of activating resting T-cells in the absence of accessory cells (57, 81). More recently, the mitogenic activity of HTLV-1 has been demonstrated to require cell-to-cell contact and is restricted to infected T-cells (145). HTLV-1 infected T-cells express high levels of IL-2R (299), although most do not release IL-2 (309), and are able to induce IL-2 independent proliferation (54). The ability of HTLV-1 to initiate T-cell activation, combined with its ability to induce the expression of genes required during this process, may permit the virus to both initiate and maintain the lymphoproliferative process. The route by which HTLV-1 initiates T-cell activation has not been thoroughly defined. Competitive blocking experiments have shown that monoclonal antibodies directed against the CD2 receptor can efficiently block HTLV-1-induced proliferation (55) and CD2 and its ligand LFA-3 are essential for HTLV-1-mediated T-cell activation (145). Thus the expression of CD2 is relevant to HTLV-1 infection and possibly to subsequent viral expression.

Intracellular Effects of HTLV

By far the most actively studied area of HTLV-1 infection has concerned the intracellular interactions of the viral-encoded p40x or *tax* protein. *Tax* is one of the *trans*-activators of HTLV-1 transcription, acting indirectly via as yet undefined cellular intermediates on *cis*-acting elements upstream of the TATA box of the proviral LTR

promoter. There are several transcriptional control elements contained within the LTR which are responsive to *tax* and other activation signals (Figure 2). Of particular interest is a 21 bp *tax*-responsive element (TRE-1) reiterated three times within the LTR (29, 251, 279, 280, 306). Within each TRE-1 is an octameric core sequence similar to the cyclic-AMP-responsive element (CRE) identified in cellular genes (123). Although the LTRs of HTLV-1 and HTLV-2 share only 60% homology (274), the TRE-1 sequences are highly conserved and are present in the same number in the HTLV-2 LTR (262, 273, 281). However, studies of the transcriptional regulation of HTLV-2 to agents other than the *tax* protein are lacking. In addition to TRE-1 sequences, the integrity of two imperfectly conserved tandem 51 bp repeats, each containing one 21 bp repeat, appears to be required for optimal transactivation of HTLV-1 by *tax* (243). The response of one 21 bp repeat to *tax*-mediated transactivation is further enhanced by a sequence of 4 pentanucleotide repeats (TRE-2) located between the second and third TRE-1 sequences (193). A 25 bp sequence (TRE-2S) within the TRE-2 is required for cooperative *tax trans*-activation. Mutagenesis studies indicate that Ets and NFκB binding sites in the TRE-2S are dispensible to the cooperative effect (290). HTLV-1 transcription is upregulated by phorbol ester, which activates PKC. This response is determined by a 60 bp element which overlaps with one 21 bp repeat and requires the integrity of the two 51 bp repeated elements for optimal response to phorbol ester (243). Basal LTR activity is also dependent upon the presence of sequences located within the U5 region of the LTR (137). The presence of an Ets responsive element (ETS RE) in the LTR is required for inducible enhancer function but is not essential for *tax*-mediated *trans*-activation (36).

Several proteins have been identified which bind to the LTR. These include TIF-1 (194), members of the ATF or CREB family (182, 288), SP-1 (224), HEB1 and HEB2 (208) and the c-*ets*-1 (22) and c-*jun* (124) proto-oncogene products. Recently several new proteins were identified to bind to the LTR including Thp-1 and -2, members of the GL1 family of proteins (290), a human Ets family member, Elf-1 (36) and the NFκB protein PrdII-BF1 (261). Although *tax* has not been shown to bind directly to the LTR, it can bind

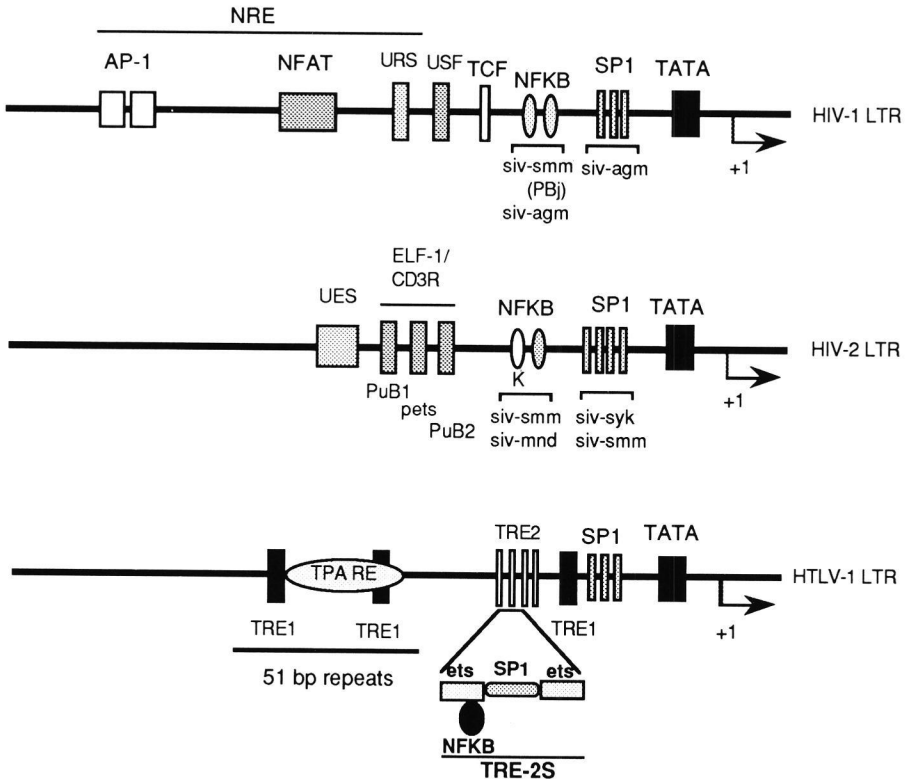

Figure 2. *Organization of the LTRs of HIV-1, HIV-2 and HTLV-1. Similarities between NF κB and SP1 elements of HIV and SIV strains are indicated and further discussed in the text. RE, responsive element; URS, upstream response sequence; USF, upstream suppressor factor. Other sites are defined in the text. +1 indicates the start site of transcription.*

indirectly to the TRE-2 via the 36 kDa TIF-1 protein isolated from HeLa nuclear extracts (194).

In addition to induction via *tax* and phorbol ester, the HTLV-1 LTR can also be induced by forskolin, an activator of adenylate cyclase (214). This cAMP-specific induction is mediated via the octameric CRE within the TRE-1 elements of the LTR and is dependent upon the availability of protein kinase A. Site-directed mutagenesis of the LTR has revealed that although some sequences required for cAMP-induced activation of HTLV-1 are also required for *tax*-mediated transactivation, the two mechanisms are independent

(129). The pathways by which *tax* exerts its *trans*-activating function, although not completely understood, appear to be independent of both PKC (243) and cAMP pathways (241) and suggests that *tax* acts independently of the other known activation pathways utilized by the HTLV-1 LTR. Although HTLV-1 has been described as a mitogenic agent, little has been reported of the response of the virus itself to T-cell activation signals. Expression from the LTR has been shown to be upregulated by phorbol ester (243) but studies of activation via cell surface T-cell receptors are lacking.

Transcriptional activation by *tax* has also been observed for several cellular and viral genes. *Tax* acts indirectly through an NFκB element in the activation of the HIV-1 promoter (9, 276), the promoters of the interleukin-2 receptor gene (46, 164, 227), TNF-α gene (4), TNF-β and immunoglobin κ light chain genes (173), the human vimentin gene (169), and the c-*rel* (167) and c-*myc* (59) proto-oncogenes. *Trans*-activation by *tax* is also mediated through CREs in the promoters of the human globin genes (72) and the c-*fos* proto-oncogene (75). Thus *tax* appears to be capable of interfacing with different families of host cell transcription factors to effect the *trans*-activation of viral and cellular genes. *Tax* has been shown to bind to the NFκB precursor, p105 (107), and to possess distinct domains for both transcriptional activation and enhancer specificity (76). While NFκB p50 or p65 subunit activites are not present in HTLV-1-infected cells, c-*rel* activity has been detected (167). *Trans*-activation by *tax* has also been reported for other cellular genes including those coding for GM-CSF (219), parathyroid hormone-related protein (60), MHC I (257), TGFβ (144), proenkephalin (127), and c-*sis* (246). Interaction of *tax* with cellular transcriptional factors may provide a mechanism for the activation of cellular genes involved in the transformation observed in HTLV-1 infected cells.

IV HIV INFECTION

Extracellular Viral Effects

HIV infects T helper-lymphocytes via the CD4 receptor, and AIDS is characterized by a loss of this cell population. While CD4+ T-cells appear to be a major target for infection, CD4- cells have also been shown to become infected (31, 33, 100, 314). CD4 internalization does not occur during HIV infection, nor are CD4 regulated signal transduction events required for virus entry (228). Cells of the mononuclear macrophage lineage may also be infected and have been identified as a major reservoir of virus during infection (157, 166, 179, 233). During chronic infection, CD4 mRNA expression is down-regulated (111) resulting in a loss of the CD4 glycoprotein on the cell surface. A reduction in virus expression results in restored CD4 expression (269). This taken together with the observations that some cell lines expressing high levels of CD4 are not productively infected by HIV (66, 140) and that some CD4- cells may become infected, suggests that an alternate pathway(s) may exist to mediate HIV entry. In the case of CD4- cells, the *in vitro* detection of HIV production appears to be dependent upon coculture with other cells such as PBMC. In this instance, virus production appears to be upregulated by cytokines provided by the PBMC (287). Productive infection by HIV is dependent upon the integration of proviral DNA into the host genome (285, 328). Studies of HIV infection of T-cells have shown that activation of the cell is required for both entry (89) and subsequent integration of the provirus (285). In the absence of activation, the proviral DNA remains extrachromosomal and in this form may persist for up to weeks before being cleared by the cell (285).

The cytopathic effect of HIV may be due to a direct toxicity induced by viral envelope proteins. An increase in gp120 expression during infection has been shown to result in cell death (284). Cell death was also observed following the addition of gp120 to PBMC or to cultured brain cells (23, 53, 130). The expression of HIV proteins on the cell surface may induce cell death by affecting membrane permeability (53) or the toxicity may be initiated

by the interaction between gp41 and CD4, which is mediated by a tyrosine phosphorylation-dependent cellular activation pathway (43).

Intracellular Effects of HIV

The HIV-1 promoter shares several *cis*-acting regulatory elements in common with the IL-2 and IL-2R promoters, including NF-AT, AP-1, SP-1 and NFκB (Figure 2). The enhancer of the HIV-1 LTR has been localized to a twice repeated 11 bp sequence which binds the transcription factor NFκB (113, 213) and a second mitogen-inducible protein, HIVEN 86A (21). HIV-1 expression is positively regulated at the level of transcription by the virally encoded *tat* protein which interacts directly with a specific region (TAR) downstream of the transcription start site; thus the TAR sequence is present in both DNA and RNA (16, 67, 79, 102, 121, 251, 263). The proposed function of the RNA TAR moiety is to serve as a link for *tat* to the DNA transcriptional machinery (15, 264, 283). *Tat trans*-activation of the LTR, although dependent upon activation of the PKC pathway (120), is able to synergize with PKC-mediated activation of the LTR and to a smaller degree with calcium ionophore activation (276).

One of the hallmarks of HIV-1 infection is the long asymptomatic period observed before the occurrence of clinical disease. In PBLs isolated from infected individuals very little or no virus is produced. Virus expression can be upregulated by exposure of the cells to T-cell activating agents such as phorbol ester or the lectin PHA (71, 202). Thus T-cell activation may provide one of the initial signals required for the onset of productive virus expression during HIV-1 infection. Experiments employing transient transfection of human tumor cell lines with HIV-1 LTR-CAT vectors have shown that the HIV-1 LTR responds to signals induced by anti-CD3 mAb at moderate to low levels (93, 192, 293) and that this activation is mediated by NFκB (192). In contrast, Hazan and coworkers (103) reported that in human T-cell clones, although triggering of T-cells via the CD3-TCR complex induced NFκB translocation, LTR-mediated gene expression did not occur.

The LTR of HIV-2 has been reported to be less responsive than HIV-1 to some T-cell activation signals due to the presence of only one conserved NFκB site (10, 294). In contrast to HIV-1, the HIV-2 LTR contains a regulatory element (CD3R or PuB1; Figure 2) which binds a specific factor in response to stimulation of the TCR-CD3 complex (192). This response is mediated partly through the CD3R, which is also responsive to stimulation by phorbol ester and PHA. The CD3R site is purine rich and has been shown to bind Elf-1, a member of the *ets* proto-oncogene family. This factor also binds to a second purine rich site in the LTR (PuB2), and the interaction of Elf-1 with these sites is required for TCR-mediated T-cell activation (162). While the NFκB transcription factor appears sufficient for activation of HIV-1, activation of HIV-2 requires additional factors. Markovitz et al. (192) demonstrated that activation of the HIV-2 enhancer is dependent upon the cooperative function of at least four cis-acting elements. Only one of these sites, NFκB, is present in the HIV-1 enhancer. Although the CD3R/PuB1 is responsive to TCR-mediated T-cell activation, it is a relatively weak response element. This observation taken together with the presence of only one functional NFκB element in the HIV-2 LTR, may in part provide an explanation for the differences in duration of disease progression in HIV-1 versus HIV-2 infection.

The requirement for the NFκB sites in LTR-directed transcription appears to depend upon the mode of activation used. Activation of the HIV-1 LTR by phorbol ester was initially reported to be mediated via the NFκB sites (138, 213). Leonard et al. (163) demonstrated, using virus deleted for the NFκB sites, that while these sites influenced the rate of viral transcription, they were not absolutely required for the replication of HIV-1 in human T-cell lines and PBLs. Transient transfection of LTR-CAT constructs in Jurkat cells revealed that deletion of the NFκB sites resulted in only a slight impairment of the response of the LTR to either PHA or phorbol ester (276). Binding of NFκB to the LTR can be inhibited by the immunosuppressive drug cyclosporin A in cells stimulated with PHA but not phorbol ester (258). Activation mediated by anti-CD3 mAb (293) or by a combination of anti-CD3 and anti-CD28 mAb (93) is markedly reduced by cyclosporin A, but again this

was not observed in cells treated with phorbol ester nor in cells treated with *tat* (276, 293). This suggests that cyclosporin A suppressible calcium-dependent events, likely early T-cell activation events, are required for activation of the LTR via cell surface pathways. Phorbol ester-mediated activation can bypass cell-surface pathways to, as a single signal, activate the LTR. Whether phorbol ester induces constitutively active transcription factors other than NFκB to bind to the LTR has not been determined.

An intragenic enhancer element has been localized within the *pol* gene of HIV-1. Three sites homologous to the AP-1 binding site were identified within this enhancer and shown to function as phorbol ester-inducible enhancers (305). The importance of the intragenic enhancer in HIV-1 regulation has not been studied in depth, however, since the enhancer has been shown to be functional and bind AP-1, this site may be available to c-*jun*, c-*fos* and related transcription factors which are up-regulated during T-cell activation.

During HIV infection, the expression of several cytokines by some cells is enhanced. Although the effect of HIV infection on cytokine production is not as yet completely understood, the effect of cytokines on HIV replication has been well studied. The cytokines produced by T-cells, macrophages and by HIV-infected cells act upon specific cellular transcription factors which bind to the responsive elements within the LTR to mediate virus transcription. HIV-1 expression has been shown to be upregulated by IL-1, TNFα and IL-6 (11, 56, 116, 147, 153, 199, 231, 308). The effect of extracellular cytokines on HIV-1 LTR-mediated expression has been studied using reporter vectors in transiently or stably transfected human T-cell lines. Using HIV-1 LTR-CAT vectors deleted for the NF-AT or NFκB sites, and mutant viruses with the same LTR deletions, Lu and colleagues (176) found that LTR-directed expression and virus transcription in response to TNFα or phorbol ester was enhanced in the absence of either of these sites. Deletion of both sites resulted in no response via these pathways. In contrast, Osborn et al. (230) reported that NFκB sites were required for LTR activation by either TNFα or IL-1.

V THE CONTROL OF HUMAN RETROVIRUS EXPRESSION

Despite the responsiveness of HTLV and HIV to various pathways observed *in vitro*, infections with these viruses are marked by asymptomatic periods during which virus expression in T-cells isolated from peripheral blood is limited. Latent infection may allow viral persistence and evasion of elimination by the host. Several mechanisms of viral regulation have been described, involving transcriptional and post-transcriptional mechanisms as well as cellular versus virus-mediated regulation of expression. The dynamic interaction of these factors may contribute to the suppression of virus expression in T-cells.

Viral Factors Mediating Control of Virus Transcription

Although *tax* is a strong inducer of the HTLV-1 LTR, a second viral-encoded protein, *rex*, mediates effects on virus expression at both the transcriptional and post-transcriptional levels. The *trans*-activation of HTLV-1 and HTLV-2 by *tax* is augmented by *rex* which acts via a *rex* responsive element (RxRE) located within the 3' LTR (19, 252). This interaction favours the expression of viral unspliced and singly spliced mRNAs encoding *gag*, *pol* and *env* and, in addition, *rex* promotes the transport of these transcripts to the cytoplasm (132,143). However, an over-expression of *rex* is inhibitory to *tax*-mediated *trans*-activation, resulting in a feed-back control of viral gene product expression which may be an important factor in the establishment and/or maintenance of a semi-latent state. The expression of HIV-1 requires a direct binding of the *rev* protein to its RNA target sequence, the *rev* responsive element (RRE), which favours the expression of singly spliced and unspliced mRNAs encoding viral structural proteins (63, 96, 185). As in the case of *rex*, a threshold level of *rev* expression is required for optimal *rev* function (238). Structural gene expression depends upon the sequential binding of multiple *rev* monomers to the target RRE (184). Thus, in contrast to over-expression of HTLV-1 *rex*, an under-expression of the *rev* protein may limit virus expression.

A negative regulatory element (NRE) is present in the U3 region of the HIV-1 LTR (Figure 2). Deletion of this element results in an increase in LTR-directed gene expression (251). DNase foot-printing revealed that nuclear factors bind to this region, including the viral-encoded *nef* protein (2, 218). The *nef* protein has been described as a negative regulatory protein based on *in vitro* experiments which demonstrated that molecular clones of HIV-1 containing deletion or point mutations within *nef* replicated to higher titers than wild-type clones bearing intact *nef* sequences (177). *Nef* has demonstrated pleiotropic functions depending on differences in both *nef* sequence and the particular cell type used in *in vitro* experiments. In T-cells, an inhibition by *nef* of NFκB induction has been reported (217). In addition, antigen receptor induction of IL-2 mRNA is prevented in T-cells expressing *nef* (178). Anti-*rev* activity associated with *nef* expression in T-cells has also been reported (166). Not all investigations have found *nef* to be suppressive to virus expression (97, 142), which may be partly explained by the observation that highly pathogenic HIV-1 strains are not susceptible to *nef*-mediated suppression (30).

Reports to date have not described a NRE within the LTR of HTLV-1, nor proteins which may down-regulate LTR-mediated gene expression. The pX region of the HTLV-1 genome, which encodes *tax* and *rex* proteins, contains an additional 8 highly conserved open reading frames (27). While 5 of these open reading frames specified proteins *in vitro*, the proteins were not produced *in vivo*.

Examination of HTLV-1 sequences from the leukemic cells of ATL patients revealed that approximately 32% of these patients carried detectable levels of deleted HTLV-1 genomes (151). The pX region and the 3' LTR were intact in these deleted viruses. Whether the deleted HTLV-1 genomes are present in asymptomatic patients has not been reported.

The HIV-1 LTR bears duplicated NFκB sites and this feature is shared with SIV derived from African green monkeys (SIVagm) and an acutely lethal strain derived from sootey mangabeys (SIVsmm/PBj). Similar to the HIV-2 LTR, SIV strains derived from sootey mangabeys and mandrills (SIVmnd) contain only one NFκB element within the

LTR. In addition, in LTRs derived from SIV isolates of Sykes monkeys (SIVsyk) the NFκB site is uniquely absent (108). Differences in the number of NFκB elements may contribute to variations observed in viral replication rates and disease progression. However, the significance of these sites *in vivo* has not yet been determined. Variation within the LTR of HIV-1 has also been noted for the Sp-1 and TCF-1α binding sites. In viral isolates of HIV-1 infected individuals, Koken and co-workers (150) observed that variants containing an additional Sp1 site had higher promoter activity and higher viral replication rates *in vitro*. TCF-1α is a conserved motif occurring upstream of the NFκB elements in the HIV-1 LTR. Duplication of TCF-1α in the LTR of a HIV-1 isolate was associated with a small reduction in virus production *in vitro* (150). Thus variation in NFκB, Sp1 or perhaps other LTR elements may contribute to differences in virus expression which may subsequently influence virulence and disease progression. Differences in NFκB and Sp1 number in the LTRs of HIV and SIV strains are summarized in Figure 2.

T-cell-Specific Cellular Events Influencing Retrovirus Expression

Human retroviruses require T-cell activation for integration of proviral DNA into the cellular genome (89, 285, 328). Due to the transient nature of T-cell activation, the cell returns to a quiescent or G_0 state. In the absence of cellular proliferation, expression of the integrated provirus is expected to be minimal. Subsequent re-activation of the infected cell might provide activation of productive infection as has been shown for HIV-1 infected cells cultured *in vitro* (99, 329). Expression of HTLV-1 in ATL patients is minimal (115, 146) despite the activated phenotype of the infected cells.

The frequency of HTLV-1-specific CD8+ cytotoxic T-lymphocytes (CTL) in HAM/TSP patients is markedly higher than in asymptomatic patients (61). Higher frequencies of CTL's may correlate with the higher level of virus expression in HAM/TSP which is not observed in ATL (148). In contrast, CD8+ lymphocytes are able to suppress

HIV-1 replication *in vitro* (24, 297, 312). The inhibition of HIV-1 replication is not due solely to CD8+ cytotoxic T-lymphocytes as the inhibition is observed in the absence of CD8+-mediated CD4+ T-cell elimination (181, 310, 311, 312, 317). A CD8+ T-cell-specific soluble factor is involved in the inhibition of HIV-1 replication (24) which is mediated at a point before RNA transcription (166).

Other mechanisms which may mediate control over gene expression include DNA hypermethylation which has been observed for both HIV-1 (255) and HTLV-1 (37, 149). In the case of HIV-1, inhibition of expression due to hypermethylation is overcome by a combination of *tat* plus phorbol ester (254) and the subsequent induction of NFκB (14). The site of integration of the HIV-1 provirus in the cellular genome has also recently been reported to inhibit HIV-1 gene expression (316).

Scope of this thesis

The studies presented here concern the role of T-cell activation pathways in human retrovirus expression, with particular reference to HTLV-1. The thesis can be subdivided into two areas of study. In the first section, two novel assay procedures were developed. A rapid assay of HTLV-1 LTR-mediated gene expression is described in Chapter 2. The assay is based on a LTR-β-galactosidase vector which was stably integrated into the genome of HeLa cells. Chapter 3 concerns the study of the *in vitro* cellular tropism of HTLV-1 using a novel assay system employing a recombinant viral vector.

The second section describes the role of T-cell activation in retroviral expression and the alterations in T-cell function in infected cells. The integrated assay described in Chapter 2 was a valuable tool used in the studies which are described in Chapters 4 and 5. The assay was duplicated in Jurkat T-cells using both the HIV-1 LTR and HTLV-1 LTR for investigations of how integrated retroviruses respond to cellular activation, as is described in Chapter 4. T-cell activation signals studied included triggering at the cell surface and direct activation of transduction pathways using phorbol ester and calcium ionophore. In

Chapter 5, the aberrant response of HTLV-1 LTR-directed gene expression to the calcium ionophore ionomycin was studied using inhibitors of calcium-dependent activation pathways, to further elucidate this apparent mechanism of HTLV-1 latency.

Chapter 6 describes the study of apoptosis induction in HTLV-1 infected cells. This study was performed to examine the cellular pathways which underly the protective role of HTLV-1 infection against apoptosis and which may contribute to the expansion of a dysregulated T-cell population.

In Chapter 7 the cumulative findings of the preceding chapters are discussed and integrated to present a model of disease progression to mature T-cell cancer.

CHAPTER II

CYTOCHEMICAL ANALYSIS OF HTLV-1 REGULATED β-GALACTOSIDASE EXPRESSION USING A NOVEL INTEGRATED CELL SYSTEM

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SUMMARY

To develop a reporter system to study the response of an integrated retroviral LTR and cellular and viral events which influence transcription, the 5' LTR of HTLV-1 was coupled to the *Escherichia coli* β-galactosidase gene (lacZ). This construct was assembled within a vector containing the neomycin resistance gene controlled by the SV40 promoter, and introduced into HeLa cells. Expression from the LTR in one clone was upregulated by positive regulators of HTLV-1 expression, including 12-O-tetradecanoylphorbol-13-acetate and the HTLV-1 *trans*-regulatory protein (*tax*), as has been previously reported using transient transfection assays. This method proved to be a rapid and reproducible assay for the measurement of integrated viral LTR activation in a single cell system.

INTRODUCTION

Human T-cell leukemia virus 1 (HTLV-1) is implicated as a principal etiological agent of adult T-cell leukemia/lymphoma, a malignancy of mature helper T-lymphocytes (106, 236, 323). The virus persists in an integrated and relatively quiescent state for the majority of the hosts life cycle. The expression of HTLV-1 is controlled at the transcriptional level via the long terminal repeat (LTR) which contains response elements for both viral and cellular factors. HTLV-1 expression is enhanced by the virally encoded p40x (*tax*) protein which acts indirectly upon a 21 bp *tax*-reponsive element (TRE) reiterated 3 times within the LTR (29, 280, 306). Within the TRE is a core sequence highly homologous to the cyclic AMP response element (CRE) found in cellular genes (123). The response of the LTR to phorbol ester-initiated activation is determined by a 60 bp element which overlaps

with one TRE (243).

Expression studies of inducible promoter elements rely heavily on transient transfection and chloramphenicol acetyl transferase (CAT) assays. Although CAT has proven to be an excellent assay of gene expression, it is limited to lysates of cell populations and does not provide information on the single-cell level. The lacZ gene of *E. coli* encodes a bacterial enzyme, β-galactosidase (lacZ), which is absent from mammalian cells. Previous reports have demonstrated the utility of the lacZ gene in studies of cell differentiation (91, 256), tumour development (171), expression of developmental genes in transgenic mice (6, 87, 256, 152), expression from cellular promoters/enhancers (68, 136) and viral *trans*-activation (3, 64, 249). LacZ can be sensitively and conveniently measured in cultured mammalian cells using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) which, when cleaved by lacZ, produces a blue halogenated indolyl derivative. Blue cells are visualized by low magnification microscopy (x20). We report on the construction of an HTLV-1 reporter plasmid in which the viral LTR directs the synthesis of lacZ, and the stable introduction of this plasmid into HeLa cells for the purpose of studying the inducibility of an integrated HTLV-1 LTR to *trans*-activating signals. *Trans*-activation of the LTR can be measured by an increase in the number of blue cells over untreated cells.

RESULTS AND DISCUSSION

The HTLV-1 LTR-lacZ-neo plasmid (Figure 1) was constructed by inserting the 654 bp SmaI/PstI HTLV-1 LTR fragment of pIBI HTLV-1 Rsa/Rsa (provided by E. Gilboa, Memorial Sloan-Kettering Cancer Center, New York) upstream of lacZ into the blunted Pst I site of the pSDKlacZ polylinker by blunt-ended ligation. The LTR fragment is -322 - +332 bp relative to the CAP site. The plasmid pSDKlacZ was provided by Janet Rossant

(Division of Molecular and Developmental Biology, Mount Sinai Hospital Research Institute, Toronto, Canada). The vector was modified by incorporation of the neomycin resistance gene, under control of the SV40 promoter, downstream of the lacZ gene. This plasmid was introduced into HeLa cells (Catalog number 153; AIDS Research and Reference Reagents Program) by calcium phosphate precipitation (90, 232) and neomycin resistant clones were isolated after culturing cells in the presence of G418 (Gibco-BRL; 1 mg/ml) in Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories).

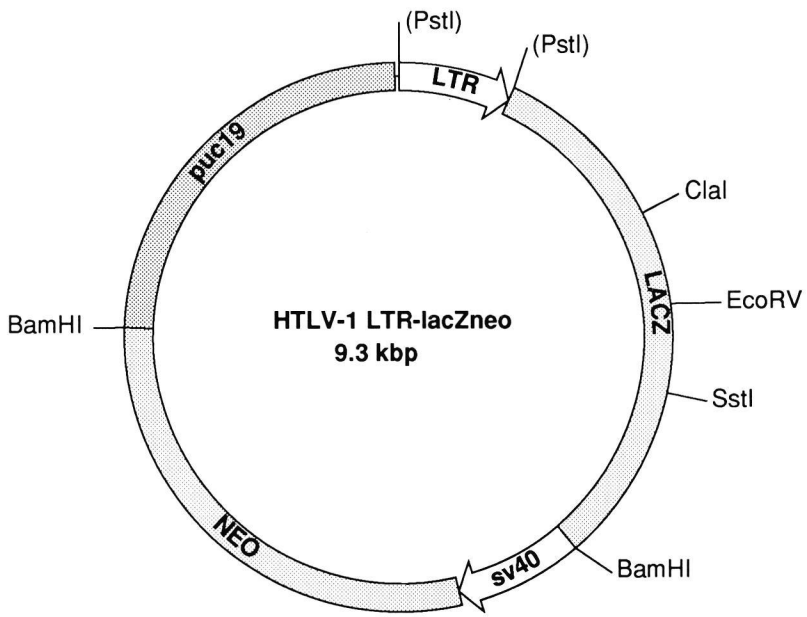
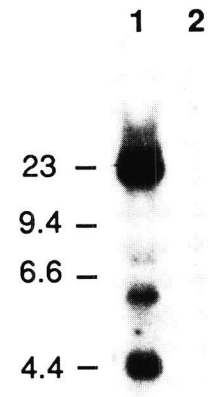


Figure 1. *Structure of pHTLV-1 LTR-lacZ-neo*

Four neomycin resistant clones were obtained and screened for lacZ expression in response to treatment with the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA; Sigma). Cells (3 x 10^5) seeded in 25 cm^2 flasks were cultured in DMEM containing 25 ng TPA per ml for 18 hours. Following fixation and X-Gal (Gibco-BRL) staining (87), blue cells were observed under low magnification. Two clones expressed no lacZ activity regardless of TPA treatment, one expressed low levels of lacZ following TPA treatment, and one clone (A-1) expressed low levels of lacZ prior to treatment and a measurable increase in expression following TPA treatment. Integration and copy number of the vector in clone A-1 were confirmed by Southern blot analysis. Figure 2 shows a Southern blot of EcoRV-digested DNA of this clone hybridized with a ^{32}P-labelled BamHI/HindIII fragment of pSDKlacZ. The lacZ sequence is cleaved once with EcoRV and thus would reveal 2 bands per copy. Hence the 4 bands seen in lane 1 confirm a copy number of 2 in this cell line.

Figure 2. *Southern blot hybridization of clone A-1. Lane 1, EcoRV digest of clone A-1 DNA; lane 2, control untransfected HeLa cell DNA. Molecular size markers (kbp) are indicated.*



The response of clone A-1 to different *trans*-activating factors was then tested with subsequent detection of lacZ using X-Gal. Cells (3 x 10^5) were seeded in 25 cm^2 culture flasks 24 hours prior to treatment. As indicated in Figure 3A, uninduced lacZ activity in unstimulated cells was approximately 0.06% of the total population of cells. This activity was generated by the integrated plasmid as blue cells were not visible following X-Gal staining of untransfected HeLa cells. LacZ expression of A-1 cells measured 48 hours following transfection with pRSV*tax* was similar to that obtained following 18 hours of TPA stimulation, resulting in a 3 fold increase in the number of blue cells over basal levels. The vector pRSV*tax* is the HTLV-1 equivalent of pBLV*tax* (50). The incorporation of the calcium ionophore ionomycin (Sigma; 2μM) in the medium during TPA treatment of the cells resulted in a decrease in the response of the LTR to TPA. The response of A-1 cells to combined treatment with *tax* and TPA demonstrated an additive effect, consistent with the findings of Radonovich and Jeang (243) that *tax*- and TPA-mediated activation occur via different mechanisms. Transfection with pCS-HTLV-1 (32), which expresses the full viral genome, resulted in higher lacZ expression than that obtained with *tax* alone. This higher level of *trans*-activation may be due to a more efficient production of *tax* by the viral promoter in its context of the entire cDNA. Alternatively, this effect may result from complementation of *trans*-activation or stabilization of lacZ mRNA by other virally encoded proteins. Incubation of the cells with supernatant of the HTLV-1-producing human T-cell line, MT-2, or direct coculture with MT-2 cells (48 hour incubation) did not produce an increase in lacZ expression, suggesting that HeLa cells may not produce the necessary factors required for productive HTLV-1 infection. This interpretation is supported by infection experiments performed in this laboratory which show that HeLa cells are not susceptible to infection by HTLV-1 (manuscript in preparation).

Although the level of lacZ expression is low with respect to the total population of cells, the results are highly reproducible. To provide for more rapid quantitation, the assay was adapted to one ml culture wells (16 mm diameter) using 3 x 10^4 cells per well (Figure

3B). Uninduced lacZ activity was slightly higher in this assay at 0.1%. Treatment with TPA resulted in an approximate 3-fold increase in the number of blue cells. Ionomycin again clearly inhibited the response of the LTR to TPA. These results compare well with those obtained using a 10-fold higher number of cells (Figure 3A).

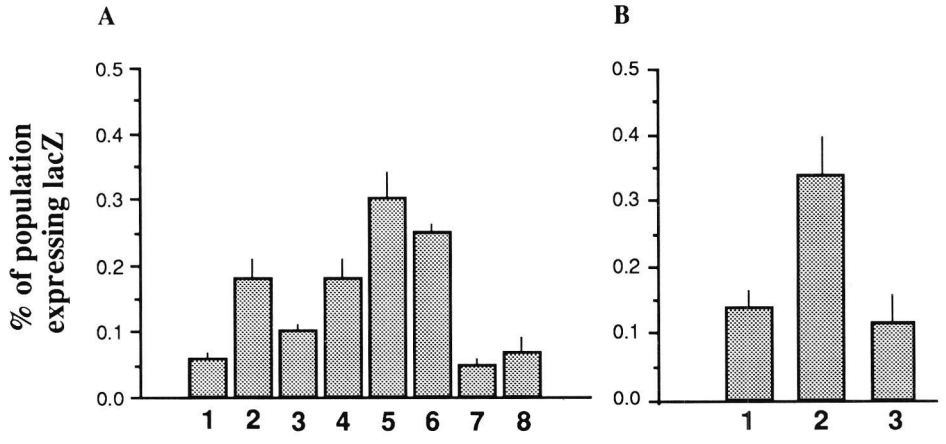


Figure 3. *(A) Response of A-1 cells to trans-activating signals as detected by X-Gal staining of 3 x 10^5 cells seeded in 25 cm^2 flasks. Treatments: 1, none; 2, TPA; 3, TPA plus ionomycin; 4, transfection with pRSVtax; 5, TPA plus tax; 6, transfection with pCS-HTLV-1; 7, incubation with MT-2 supernatant; 8, co-culture with 2 x 10^6 MT-2 cells. In the case of tax transfected cells, treatment with TPA was initiated 40 hours post transfection for 18 hours. (B) X-Gal staining of 3 x 10^4 A-1 cells seeded in 16 mm culture wells. Treatments: 1, none; 2, TPA; 3, TPA plus ionomycin. Control cells were counted by trypan blue exclusion at the time of X-Gal staining to provide an accurate determination of the total number of cells in each treatment group. Data is presented as the percent of cells expressing lacZ of the total population of cells. The values shown are representative of 4-8 independent assays.*

In this report we demonstrate that lacZ expression directed by an HTLV-1 LTR provides a simple and rapid nonradioactive assay of LTR activation. One advantage inherent to the use of lacZ as a reporter is the variety of substrates that are available for its assay, including luminescent and fluorescent assays. In the case of fluorescence

measurement, fluorescein detection following staining with FDG (fluorescein di-β-D-galactopyranoside), used in conjunction with flow cytometry, provides a single cell assay which can determine the proportion of cells expressing lacZ and the level of lacZ activity in individual viable cells. The response to *trans*-activating signals of an integrated HTLV-1 LTR-lacZ-neo vector in human T-cells using FDG detection of lacZ are currently being determined. Investigations of HTLV-1 regulation using an integrated system may provide a more accurate understanding of pathways critical to latency and expression. As HeLa cells are not susceptible to infection with HTLV-1, this system may provide a useful tool for screening putative clones of the HTLV-1 receptor by the acquisition of infectability. Infection could be monitored by lacZ production.

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CHAPTER III

DETECTION OF HUMAN T-CELL LEUKEMIA VIRUS 1 PERMISSIVE CELLS USING CELL LINES PRODUCING SELECTABLE RECOMBINANT VIRIONS

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SUMMARY

A selectable retrovirus vector based on a full length HTLV-1 provirus clone, pCS-HTLV-1, was constructed by replacing the coding regions for *tax*, *rex* and the 3' region of *env* with the prokaryotic neomycin resistance gene under the control of the CMV promoter. This vector, pHTLV-1-CMVneo, was transfected into uninfected and HTLV-1 infected human lymphocytes and fibroblasts. The production of recombinant virus by these cells was measured by the transfer of G418 resistance to target cells. Infection of target cells showed a preference for human T-lymphocyte cells in addition to two human fibroblast cell lines, Hos7 and RD4, and the African green monkey kidney cell line, Cos7. RD4 cells had previously been shown to be resistant to HTLV-1 infection. Infection could be measured within 2-3 weeks in all cell types. Because this system provides a measure of productive HTLV-1 infection, it can be used not only for the quantitative measurement of *in vitro* tropism, but also as a model to facilitate molecular studies of the natural events of HTLV-1 infection.

INTRODUCTION

Human T-cell leukemia viruses have been isolated from several forms of mature T-cell malignancies (35, 141, 236, 313, 323). The most frequent syndrome from which clonally integrated HTLV-1 is isolated is adult T-cell leukemia/lymphoma (ATLL) characterized by a rapidly progressing non-Hodgkins lymphoma with mature T-cell phenotype, $CD4^+$, Tac^+ (101), frequently with bone marrow and cutaneous involvement, concurrent leukemia and hypercalcemia (210). Although HTLV-1 displays varied cellular tropism *in vitro*, it has been associated predominantly with human malignancies of the

CD4+ T-lymphocyte population (101, 248). This *in vivo* tropism may be the result of restrictions imposed at various virus life cycle stages.

In experiments presented here, HTLV-I infection was monitored using a recombinant provirus containing a selectable marker. This strategy has been used successfully to investigate the *in vitro* cellular tropism of bovine leukemia virus (BLV) (50), and allows measurement of infection which is independent of virus-mediated gene expression. The system allows for the measure of productive HTLV-1 infection of susceptible cells requiring natural events such as uncoating, reverse transcription and integration which occur subsequent to binding and penetration.

RESULTS AND DISCUSSION

The lymphocytic cell line CS-1 expresses three intact HTLV-1 proviruses (175, 187). One of these proviruses was cloned into pUC19 to generate the vector pCS-HTLV-1 (Figure 1) which has previously been shown to be functionally active (32). In addition, we have shown that pCS-HTLV-1 is able to upregulate HTLV-1 LTR mediated reporter gene expression following transient transfection into a HeLa cell line stably integrated with an HTLV-1 LTR-lacZ vector (44). The vector pCS-HTLV-1 was modified, by the incorporation of a selectable marker, to facilitate studies of HTLV-1 infection. The coding regions of *tax*, and *rex* plus the 3' region of *env* were removed from pCS-HTLV-I by restriction endonuclease digestion with *Sal* 1 which cleaves at 5694 bp in *env* and *Sca* 1 which cuts at 8281 bp in *tax*. This region was replaced with the prokaryotic neomycin resistance gene (neo), under the control of the CMV promoter, to generate pHTLV-1-CMVneo.

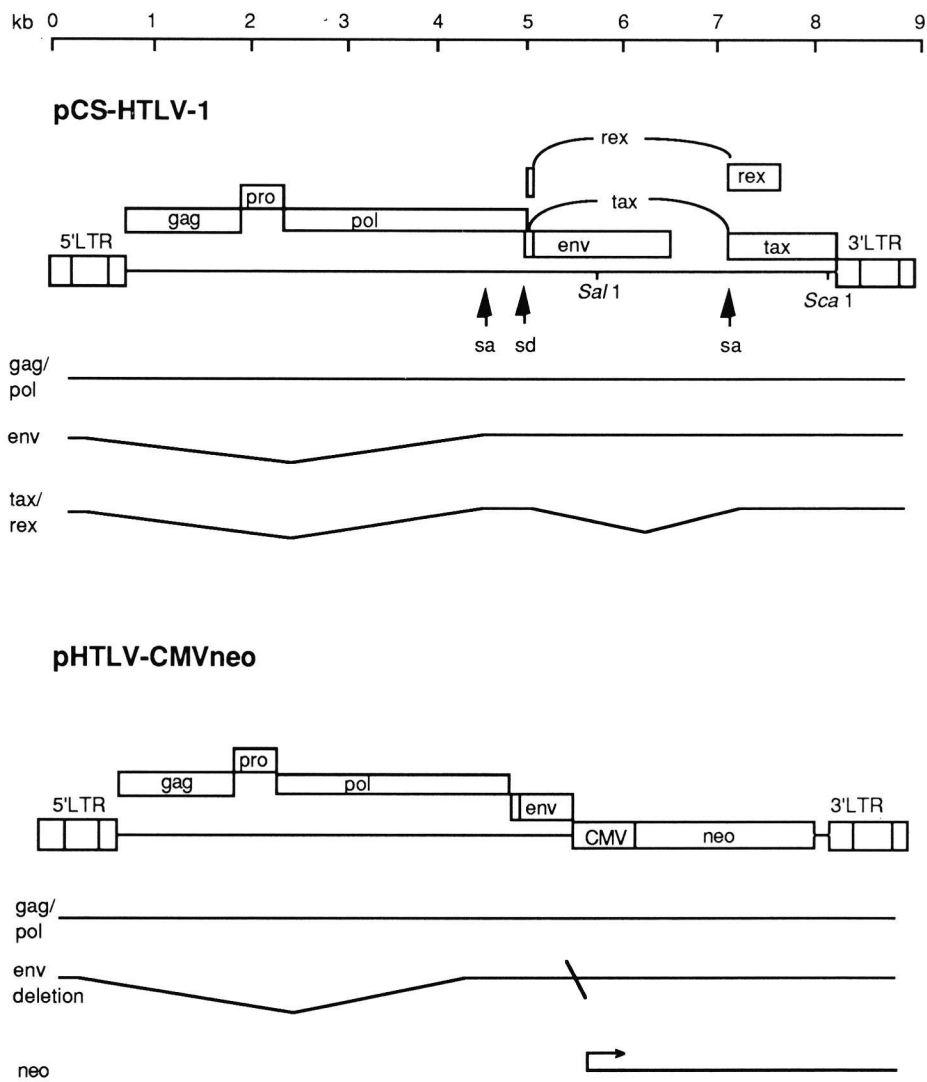

Figure 1. *Structural organization of the vectors pCS-HTLV-1 and pHTLV-1-CMVneo and transcripts produced. pHTLV-1-CMVneo was derived from pCS-HTLV-1 as described in the text. Abbreviations: sa, splice acceptor; sd, splice donor. Full length, singly- and doubly-spliced transcripts are produced by pCS-HTLV-1 and are translated to provide gag-pol, env, and tax-rex proteins. The absence of the 3' sa site in pHTLV-1-CMVneo prevents the production of a doubly-spliced transcript. Env, tax and rex transcripts are not produced by the vector. The neo transcript generated is approximately 2 kb.*

The HTLV-1-CMVneo vector was transfected into RD4 (human rhabdomyosarcoma) cells by calcium phosphate precipitation (90). Cells were maintained in Dulbecco's modification of Eagles medium supplemented with 10% fetal calf serum. Selection with G418 (Gibco-BRL; 1 mg/ml) was initiated 48 hours following transfection. RD4 clones resistant to G418 were expanded in culture. The clones were then transfected with the *env* and *tax* expressing vector pRSV-*env/tax* to provide the structural and regulatory proteins (*env* and *tax*) required for recombinant virus packaging. The transfected cells were tested for their ability to confer G418 resistance to Jurkat cells during co-cultivation. Prolonged culture was required for only a few Jurkat cells to acquire G418 resistance (data not shown), presumably due to a low titer of recombinant virus production.

To provide for more efficient production of recombinant virus, pHTLV-1-CMVneo was transfected into the HTLV-1 producing cell line Hos7 by calcium phosphate precipitation. Ten G418 resistant Hos7 clones were selected and expanded in culture. Six of the ten clones were examined by Southern analysis and found to contain integrated recombinant provirus. One of these six clones was selected for further study on the basis of a more efficient transfer of G418 resistance to Jurkat, MT4, and C8166 target cells using cell-free infection (supernatant filtered through 0.22 μ). The efficiency of this transfer was markedly higher than was obtained with RD4CMVneo cells transfected with pRSV-*env/tax*. To determine which cell types could be infected by HTLV-1, a panel of cell lines of varying types and species were infected with supernatant of the Hos7CMVneo clone. Infection was performed for 24 hours using 2 x 10^6 target lymphocytes or 3 x 10^5 target fibroblasts. In infection experiments, target cells were cultured in 5 ml of filtered Hos7CMVneo supernatant or co-cultured with irradiated (25 Grays) Hos7CMVneo cells. In co-culture experiments, Hos7CMVneo cells were grown to 75% confluency and irradiated. Target fibroblastic cells grown to 75% confluency were trypsinized and transferred to the irradiated cells. Following infection, cells were cultured in medium containing G418 (1mg/ml). All human lymphocyte lines tested acquired G418 resistance within 2-3 weeks of infection (Table 1). Of the fibroblast lines tested, the human cells Hos

Table 1. *Transfer of neomycin resistance by Hos7CMVneo cells*

Cell Type	Source	Hos7neo cell-free	Hos7neo cell-associated
Lymphocytic			
Jurkat E6-1	human T-cell	+	+
MT-4	human T-cell	+	+
C8166	human T-cell	+	+
Hut78	human T-cell	+	nd
APD	human B-cell	+	+
Fibroblastic			
Hos	human osteosarcoma	+	+
HeLa 669	human cervix	-	-
Cos-7	African green monkey kidney	+	+
G71A	bovine skin fibroblast	-	-
CHO	chinese hamster ovary	-	-
NIH3T3	mouse embryonic fibroblast	-	-
U87-MG	human glioblastoma-astrocytoma	-	-
RD4	human rhabdomyosarcoma	+	+

(+) Positive transfer of neomycin resistance measured by continued growth following death of control cells during culture in media containing 1 mg/ml G418.
(-) No transfer of G418 resistance occurred.
(nd) not done

and RD4, and the African green monkey kidney cell Cos7, acquired G418 resistance following incubation with cell-free supernatant of Hos7CMVneo and by co-culture with irradiated Hos7CMVneo cells. The results shown are representative of 2-6 replicates where successful infection was measured by the continued growth of cells in G418 well after the death of control cells (cultured in supernatant of, or with cells not expressing the recombinant virus). In most cases control cells died between 6-11 days. Positive transfer of G418 resistance could be detected by 2 weeks and allowed growth of cells beyond this time point.

HTLV-1-CMVneo was also transfected into MT-2 cells which, like Hos7 cells, actively produce HTLV-1. MT-2 cells were transfected with pHTLV-1-CMVneo using the DEAE dextran procedure. Briefly, 3 x 10^7 cells were suspended in 5 ml RPMI 1640 containing 10 μg plasmid DNA, 250 μg DEAE-dextran per ml, and 50mM Tris hydrochloride (pH 7.5). Following 30 minutes incubation at 37°C, the cells were pelleted, washed twice and then cultured in complete medium for 48 hours of culture. The cells were then transferred to fresh medium supplemented with 1 mg G418 per ml. Filtered supernatant of the MT-2CMVneo clone successfully transferred G418 resistance to a panel of lymphocyte lines. Lymphocytes acquired resistance to G418 with varying kinetics over 2 weeks following 24 hours of culture with filtered supernatant of MT-2CMVneo cells and were maintained on G418 containing media for a further 3 weeks (data not shown).

Virus production by HTLV-1-CMVneo expressing RD4, Hos-7, and MT-2 cells was measured using a HTLV-1 antigen capture assay (Table 2). Provirus load was determined by Southern analysis of the DNA of each cell type and is reported as copy number in Table 2. Hos7CMVneo cells containing 3 copies of integrated provirus produced moderate levels of virus as detected by antigen capture. The efficiency of virus production was improved further in MT-2CMVneo cells, representing an approximate 3-fold increase in virus production over that of untransfected MT-2 cells. This increase may suggest a high level of virus production by the recombinant virus in MT-2CMVneo cells, however the antigen capture assay does not distinguish between wild type and recombinant viruses. While higher virus titers were produced by MT-2CMVneo cells than Hos7CMVneo cells, this does not necessarily imply a higher production of recombinant provirus by the T-cell line. MT-2CMVneo cells bear one copy of the recombinant provirus while Hos7CMVneo cells bear three integrated copies. The possibility of a recombinational event occurring, resulting in the formation of a replication competent virus containing neo and *tax* is small, but may also provide an explanation for increased virus production.

Table 2. *Quantitation of gag production in supernatant of HTLV-1 and HTLV-1-CMVneo expressing cells*

Cell Type	*gag* protein μg/L[a]	Recombinant Provirus Load (copy number)
MT-2	421	0
Hos7CMVneo	171	3
RD4CMVneo + pRSV-*env/tax*	0.5	2
MT-2CMVneo	1061	1

[a] *Production of p24 and precursor p53 proteins was measured using a Coulter Antigen Capture Assay kit.*

The specific cell-surface receptor recognized by HTLV-1 is unknown. Using vesicular stomatitis virus (VSV) pseudotypes bearing HTLV-1 glycoproteins, it was demonstrated that somatic cell hybrids with human chromosome 17 were susceptible to infection, suggesting that the search for the gene encoding the receptor for HTLV-1 may be limited to 17q (282). Earlier studies have demonstrated the ability of non-lymphocyte cells to be infected *in vitro* with HTLV-1 using co-culture with virus-infected cells or by culture with cell-free virus. The human osteosarcoma cell line Hos was shown to support full virus replication using both methods (34). In addition, human endothelial cells (112) and human B cells (175, 187, 286) have also successfully supported infection with HTLV-1. Longo and colleagues (175) isolated an HTLV-1-infected B lymphocyte clone from peripheral blood of an ATL patient. However, infection of these cells may have occurred during the *in vitro* culture involved in isolation of the cells.

The data presented here extend previous investigations of the *in vitro* tropism of HTLV-1. Infection measured by the transfer of neomycin resistance occurred with all human lymphocyte lines tested. Three fibroblast cell lines, Hos7, Cos7 and RD4, acquired neomycin resistance. In a previous study, Hos7 cells were shown to be permissive to HTLV-1 infection, but the establishment of productive infection required at least 4 months

of co-cultivation with HTLV-1 infected Hos or MT-2 cells (34). In the same experiments, Clapham and colleagues were not able to demonstrate infection of RD4 cells by HTLV-1. Our results show that RD4 cells are very efficiently infected by recombinant HTLV-1 produced by two different cell types. Infection of RD4 cells has also been observed using a BLV recombinant provirus (50).

Most cell types previously tested for susceptibility to HTLV-1 infection have proven permissive for HTLV-1 binding, penetration or HTLV-1-induced cell fusion (34, 154). In addition, pseudotypes of VSV displaying HTLV-1 envelope antigens were able to penetrate a diverse array of mammalian cell types (34). While these results support the view that HTLV-1 receptors are commonly expressed on many cell types, very few non-lymphoid cell types are able to support infection. This may reflect restrictions at life cycle steps which occur following binding and penetration. We report on a system which allows for the study of early events that may prevent productive infection in these cells. In the experiments described here, a selectable recombinant provirus was used to identify cell types which support infection by HTLV-1. Our results confirm previous evidence of lymphocytes as a preferred target for HTLV-1 infection and go further to identify two nonlymphoid cell types able to support productive infection. This system provides a more rapid indicator of infectability than other methods previously described and may be useful in the selection of cells which express the HTLV-1 receptor.

Acknowledgements

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CHAPTER IV

CELL SURFACE REGULATION OF HUMAN RETROVIRAL EXPRESSION IN STABLY TRANSFECTED SINGLE CELLS

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SUMMARY

Human T-cells containing stably integrated HIV-1/ or HTLV-1/ LTR-lacZ vectors were developed for the purpose of studying the LTR responses to cell surface signals. Responses were compared to those obtained in parallel with human T-cells expressing lacZ under the control of the cellular enhancer element NF-AT of the IL-2 promoter. The effects of cellular events on the promoters of HIV-1, HTLV-1 and NF-AT were measured by the detection of β-galactosidase gene expression. The substrates fluorescein di-β-D-galactopyranoside (FDG) and 4-methylumbelliferyl-β-D-galactopyranoside (MUG) were used to measure β-galactosidase expression. Activation induced via the cell surface TCR/CD3 complex or via the CD28 receptor elicited responses from the LTR of HIV-1, however there was no additive effect on expression when both signalling pathways were triggered simultaneously. Interestingly, HTLV-1 LTR directed expression was not observed following triggering of these cell surface pathways. Activation by elevation of intracellular Ca^{2+} levels along with protein kinase C (PKC) signals was required for optimal expression of the HIV-1 LTR and the NF-AT responsive promoter, however increased intracellular Ca^{2+} was clearly inhibitory to the expression from the PKC stimulated HTLV-1 LTR. Time course experiments revealed a sustained PKC-mediated response by the HTLV-1 LTR which was detectable as early as 6 hours following stimulation. The HIV-1 LTR and NF-AT element displayed similar kinetics in response to stimulation by direct activation of PKC and Ca^{2+} pathways, with lacZ expression beginning as early as 3 hours post-stimulation. In response to activation at the TCR/CD3 complex, HIV-1 LTR-directed lacZ expression preceded NF-AT-directed lacZ expression by at least 6 hours.

INTRODUCTION

Binding of the T-cell receptor (TCR)/CD3 complex with antigen at the cell surface triggers a cascade of intracellular events which culminate in T-cell proliferation and immunologic function (7, 301). Signals from the TCR/CD3 complex are transmitted from the cell surface by a series of enzymatic steps. These involve the hydrolysis of membrane bound phosphatidyl inositol 4,5-bisphosphate by phospholipase C to yield diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (39). DAG activates protein kinase C (PKC) resulting in the translocation of the phosphoprotein to the cell membrane (17, 221), and the production of IP3 triggers a bimodal elevation of free Ca^{2+} concentration within the cell (39, 80). Both PKC activation and the generation of free Ca^{2+} ions are essential to the expression of many genes required during T-cell activation, which are activated in a hierarchical fashion (45). One of these genes, IL-2, is triggered very early in T-cell activation, responding to transcriptional factors expressed immediately in the activation process. HIV-1 expression can be detected within 2 hours following T-cell activation, and depends in part, as does IL-2, on the expression of genes falling into the immediate T-cell activation category (45). T-cell activation is required during HIV infection to enable provirus integration and subsequent expression. In quiescent cells, infection is non-productive as a result of incomplete reverse-transcription (328). Furthermore, there is great sequence similarity of regulatory elements in human retroviral LTRs to T-cell activation responsive genes. These observations suggest the participation of factors specific to activated T-cells in retroviral expression and virus production.

The measurement of ligand-induced activation of human retroviruses has been hampered by the lack of a sensitive single cell reporter assay. We have chosen to study the activation requirements of an integrated HIV-1 LTR fused to the *E. coli* lacZ gene in human Jurkat T-cells using fluorogenic assays to quantitate lacZ production. Jurkat cells were selected as a model because they phenotypically resemble resting T-cells and respond to activation signals in an IL-2/IL-2R expression-dependent fashion, as has been described in

detail in previous studies of human T-cell activation. The use of an integrated reporter assay eliminates the problems inherent in transient transfection systems, such as variability of transfection efficiency and the perturbation of cell membrane and intracellular physiology caused by transient transfection procedures previously used to study human retroviral expression.

In this study the response of a stably integrated HIV-1 LTR lacZ reporter gene to ligand- and mitogen-induced stimulation was measured by flow cytometry using the fluorogenic dye fluorescein-di-β-D-galactopyranoside (FDG) and by spectrofluorometric assay of cell lysates using 4-methylumbelliferyl-β-D-galactopyranoside (MUG). The response of the LTR was compared to 1) the HTLV-1 LTR and 2) a multimer of the binding site recognized by the nuclear factor of activated T cells (NF-AT) in the activation of IL-2 expression. NF-AT is a representative response element of one of the earliest genes triggered within the cell upon T-cell activation (45, 270). The results presented here provide information on how integrated provirus may respond to immunological signals and how these responses compare with a cellular enhancer element binding a transcriptional factor integral to T-cell activation.

MATERIALS AND METHODS

Construction of vectors

The vectors used to generate neomycin resistant cells bearing HTLV-1 or HIV-1 LTR-lacZ are shown in Figure 1. The 653 bp PstI/SmaI HTLV-1 LTR fragment of pIBI HTLV-1 Rsa/Rsa (provided by E. Gilboa, Memorial Sloan-Kettering Cancer Center, New York) was introduced into the pSDKlacZ polylinker by blunt-ended ligation to generate the vector pHTLV-I LTR-lacZ. The LTR fragment spans -322 - +332 bp relative to the CAP site. The vector pHIV-1 LTR-lacZ was constructed by inserting the HindIII HIV-1 LTR insert of the pBR322 vector pON-1 (provided by L.A. Herzenberg, Stanford University, California)

into the HindIII site of the pSDKlacZ pUC19 polylinker by sticky-end ligation. The HIV-1 LTR is derived from the ARV-2 strain (135) and spans -633 - +80 bp relative to the CAP site. Incorporation of the pSV2a neomycin resistance gene from the vector pnAZ (reference 20; obtained from L.A. Herzenberg) was at the BamHI site 3' to the lacZ gene to generate pHTLV-1 LTR-lacZneo and pHIV-1 LTR-lacZneo. The vector pSDKlacZ was provided by J. Rossant (Division of Molecular and Developmental Biology, Mount Sinai Hospital Research Institute, Toronto).

Reagents

The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma) was dissolved in DMSO to provide a 10 mg/ml stock solution. Aliquots of 10 μg/ml were prepared in culture medium and stored at -20^0 C and fresh aliquots were used in each assay. Ionomycin (Calbiochem) was prepared as a 1 mM solution in DMSO and stored at 4^0 C protected from light. The monoclonal antibodies (mAb) to CD3 (CLB-T3/3) and CD28 (CLB-CD28/1) cell surface receptors were produced by Rene van Lier at the Central Laboratory of the Netherlands Red Cross Transfusion Service, Amsterdam, the Netherlands.

Transfections

The vectors pHTLV-1 LTR-lacZneo or pHIV-1 LTR-lacZneo were introduced into human Jurkat cells by electroporation. Jurkat cells (2x10^6) were suspended in 250 μl RPMI (Gibco-BRL) supplemented with 20% fetal calf serum. The cells were mixed with 10 μg of plasmid DNA, transferred to a 0.4 cm gap electroporation cuvette (Biorad) and electroporated (250V, 960 μF) using a Gene Pulser (Biorad) with capacitance extender. Cells were cultured for 2 days in RPMI supplemented with 20% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin before selection in G418 (Gibco-BRL; 1 mg/ml). Cells were then cultured in Iscove's Modification of Eagle's medium (Flow Laboratories)

for cloning from single cells and for subsequent use in T-cell activation assays. Jurkat NF-ATlacZ cells contain a stably integrated lacZ gene under the control of three tandem repeats of the NF-AT binding site, as previously described (68). These cells were cultured in Iscove's medium containing 300 μg hygromycin B (Boehringer Mannheim) per ml of medium.

T cell activation assays

Assays were performed in 24-well Costar dishes using 6 x 10^5 cells per well. NF-ATlacZ cells were cultured with activating agents in 25 cm^2 Costar flasks. The response of NF-ATlacZ cells to activation is maximal when cultured at approximately 3x10^5 cells/ml (68). Wells and flasks were pre-coated with 1 ml of 3 μg/ml anti-CD3 in sterile PBS (1:1000 dilution of ascites) at 4^0 C for 24 hours. The coated wells were washed with PBS immediately prior to the addition of cells. Cells were added to untreated or coated wells and then cultured with activating agents for 18 hours. Anti-CD28 mAb was added to cells at 3 μg/ml. TPA was used at 10 ng/ml and ionomycin at 2 μM. LacZ expression was monitored using FDG and MUG assays. In time course experiments, 1 x 10^7 cells were cultured in 75 cm^2 flasks (Costar) coated with anti-CD3 mAb or containing TPA or TPA plus ionomycin at the concentrations mentioned above. LacZ expression was monitored using FDG at 3, 6, 12, 18, and 24 hours following stimulation.

β-galactosidase assays

In preparation for flow cytometric analysis of lacZ expression, cells were washed with staining media (PBS supplemented with 4% FCS), resuspended in 50 μl staining media and incubated on ice for 10 minutes. Following incubation at 37^0 C for 10 minutes, 50 μl of pre-warmed 2 mM FDG (Molecular Probes), prepared in distilled water, was added and the cells were mixed and returned to 37^0 C for exactly 1 minute. The loading of FDG was stopped by the addition of 1 ml ice-cold staining media containing 1 μg/ml

propidium iodide. The stained cells were incubated on ice for 30 minutes and analyzed on a FACScan (Becton Dickinson) flow cytometer. FDG-stained NF-ATlacZ cells were incubated on ice 2 hours prior to measurement as has been previously described (68).

For examination of lacZ production spectrofluorometrically, cells ($5x10^5$) were washed once with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) and resuspended and lysed in 100 µl Z buffer containing 0.1% Triton X-100. For measurement, 20 µl of 50 mM MUG in DMSO was mixed with 2 ml PBS in a quartz cuvette. Then 100 µl of cell lysate was added and mixed by pipetting up and down. Fluorescence at 376 nm was measured for one minute using 350 nm excitation with a luminescence spectrometer (LS50; Perkin Elmer). The rate of fluorescence increase was determined by linear regression analysis.

Cell cycle analysis

Cells were washed once with PBS, recovered in 0.5 ml PBS and fixed by the addition of 3 ml ice-cold 70% ethanol. The ethanol was added to the cells using a syringe fitted with a 25 gauge needle while vortexing the cells continuously. This method ensures a narrow, well-resolved G_0+G_1 peak in subsequent analysis of DNA using propidium iodide staining. At this point cells could be stored at -20^0 C. To measure cell cycle position, the fixed cells were pelletted and resuspended in PBS containing 10 µg/ml RNase and incubated at 37^0 C for one hour. The DNA was then stained by the addition of an equal volume of PBS containing 0.1% Triton-X-100 and 20 µg/ml propidium iodide. The stained cells were incubated for 30 minutes at room temperature before FACScan measurement. To determine the proportion of cells in G_2+S+M phases, a threshold was set at the right side of the G_0+G_1 peak in the propidium iodide fluorescence histogram.

RESULTS

Construction and selection of HIV-1 and HTLV-1 β-galactosidase cell lines

Transfected Jurkat cells resistant to G418 were cloned from single cells by limiting dilution and clones were then tested for lacZ expression, following phorbol ester stimulation, using FACS analysis of FDG stained cells. The percentage of lacZ+ cells was determined in a manner similar to that used by Karttunen and Shastri (136). A marker was positioned so that 90-95% of the cells in a lacZ- sample were contained below the lower boundary (to the left of the marker). Cells producing fluorescence extending beyond the marker were considered to be lacZ+. This method of measurement was chosen due to the bimodality of some cell histograms following certain stimulations.

LacZ expressing clones were selected on the basis of low background fluorescence and an increase in fluorescence following treatment with TPA. HTLV-1 clones in general showed a high level of uninduced lacZ activity and a low response to induction by TPA. Of 21 clones screened, two presented lower background and a 2-3 fold increase in lacZ activity in response to TPA treatment. Clones obtained from the HIV-1 LTR expressing cells did not present high uninduced lacZ activity. Of 24 clones screened, 3 produced a 4-fold increase in lacZ expression in response to TPA. All cell clones used in this work, including the NF-AT lacZ expressing cells, were examined by Southern analysis and were found to contain one copy of the lacZ gene (data not shown). Figure 2A and B show fluorescence histograms of responses to TPA by the HTLV-1 LTR expressing cells and to TPA plus ionomycin by cells expressing the HIV-1 LTR. Both cell lines show significant increases in fluorescence in response to stimulation, but this increase was low as compared to cellular promoter controlled integrated vectors (68, 136, 222). The NF-ATlacZ Jurkat cells, which contain an integrated vector in which lacZ expression is controlled by 3 tandem repeats of the NF-AT enhancer, do show a strong response to TPA plus ionomycin (Figure 2C) as was previously described by Fiering et al. (68).

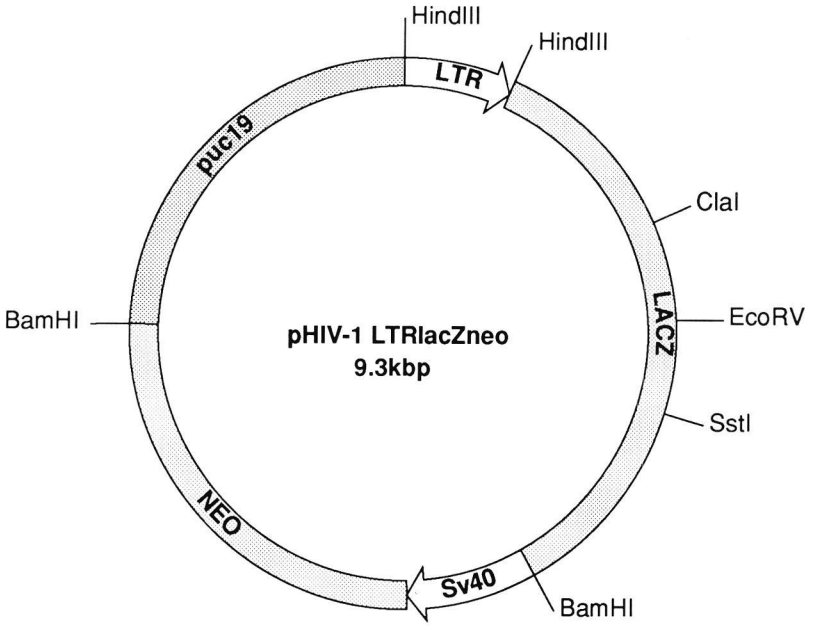

Figure 1. *Structure of the vector pHIV-1 LTR-lacZneo. The vector pHTLV-1 LTR lacZneo is identical in basic structure but contains the HTLV-1 LTR introduced at the PstI site of the pUC19 polylinker.*

Response of cell lines to T-cell activation signals

Stimulation with immobilized anti-CD3 mAb induced lacZ expression in both HIV-1 LTR and NF-AT expressing cell lines (Figure 3A) as assayed using flow cytometry of FDG-stained cells. Surprisingly, anti-CD3 stimulation elicited no response of the HTLV-1 LTR. To further determine the response of the LTRs to cell surface stimulation, cells were cultured in the presence of anti-CD28 mAb. The HIV-1 LTR responded to antiCD28 stimulation, showing a 5-fold increase in positive cells (Figure 3A). However, costimulation with antiCD3 plus antiCD28 mAbs did not result in complementation. In contrast, the HTLV-1 LTR did not respond to antiCD28 stimulation, nor was the combination of antiCD3 plus antiCD28 mAb able to induce expression. AntiCD28 mAb did

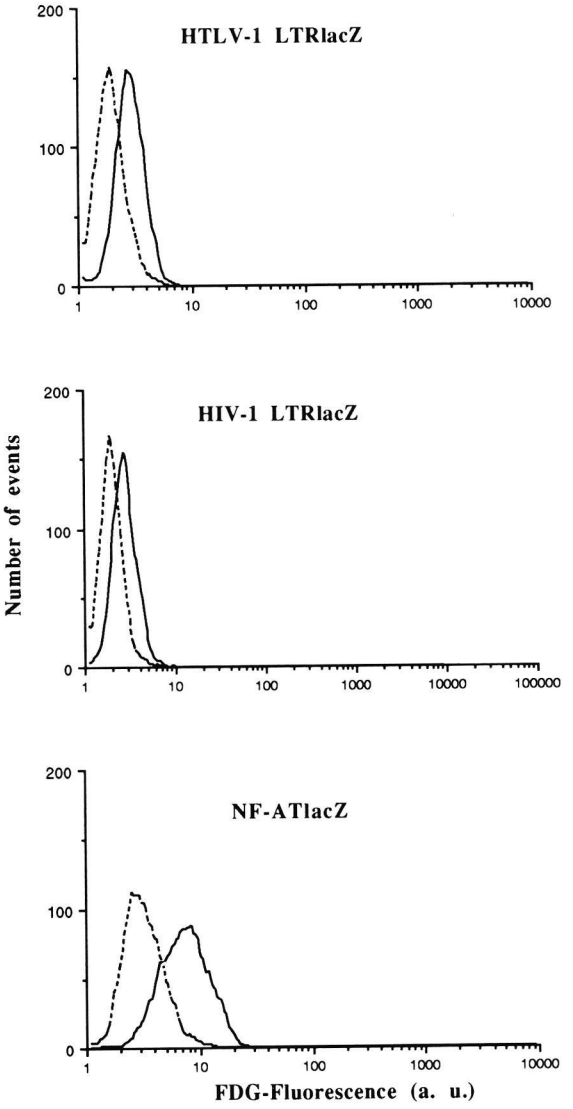

Figure 2. *Induction of lacZ in stably transfected cells.*
Fluorescein fluorescence histograms generated by FDG stained lacZ expressing cell lines before (broken lines) and after (solid lines) stimulation of (A) HTLV-1 LTRlacZ with TPA, (B) HIV-1 LTRlacZ and (C) NF-ATlacZ cells with TPA plus ionomycin. Cells were treated with activators 18 hours and fluorescence then measured by flow cytometry.

not directly induce NF-AT, nor did this treatment significantly complement antiCD3-mediated activation. The results obtained using the FDG assay were verified by MUG detection of lacZ in cell lysates following stimulation with the same panel of T-cell activation agents (Figure 3B). In all cases similar increases in fluorescence were obtained, following treatment with activating agents, using both FDG and MUG assays.

Direct activation of intracellular pathways resulted in the highest levels of lacZ production (Figure 4A). Cells expressing an integrated HIV-1 LTR responded to TPA stimulation and this response was significantly enhanced in the presence of ionomycin. As previously demonstrated (68), TPA-induced stimulation of NF-AT-directed expression is dependent upon a rise in intracellular [Ca2+] as provided by ionomycin. In contrast, the integrated HTLV-1 vector produced a 2-3 fold increase in lacZ expression in response to TPA stimulation and this response was dramatically reduced in the presence of ionomycin. The results were again verified by MUG detection of lacZ in cell lysates (Figure 4B).

Time course of induction of lacZ expression

To determine the time course of viral gene induction, lacZ expression directed by the NF-AT enhancer or HIV-1 LTR was measured over a 24 hour period following treatment of cells with either antiCD3 mAb (Figure 5A, C) or the combination of TPA plus ionomycin (Figure 5B, D). The expression of lacZ directed by the HTLV-1 LTR was measured in an identical manner, but in response to antiCD3 mAb (Figure 5E) or to TPA alone because of the inhibitory effect of ionomycin on LTR induction (Figure 5F). Following treatment with TPA and ionomycin, the HIV-1 LTR responded within 3 hours. and lacZ expression was stable until approximately 18 hours post-stimulation. In response to antiCD3 mAb, HIV-1 LTR-directed lacZ expression displayed similar kinetics but at lower levels of expression. In contrast to the HIV-1 LTR, the response of the NF-AT element to antiCD3 mAb was much slower, showing a definite lag period prior to the production of peak lacZ levels. However, in response to direct activation with TPA plus

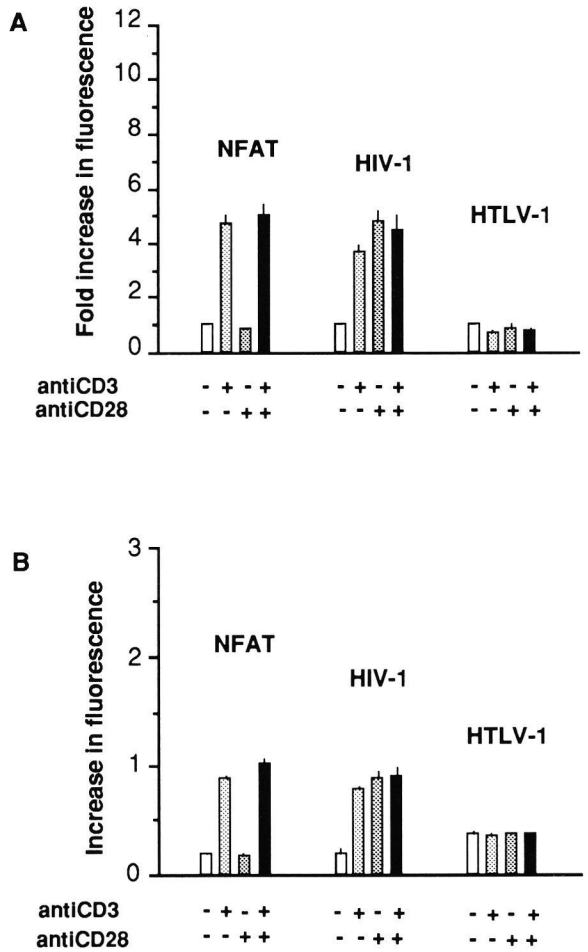

Figure 3. *LacZ expression in response to cell surface mediated activation.*
(A) LacZ expression in cells cultured for 18 hours with anti-CD3 or anti-CD28 monoclonal antibodies was measured by flow cytometry of FDG stained cells. Results are reported as the fold increase in the percentage of lacZ+ cells relative to the unstimulated control and are the mean and standard deviation of 4 independent experiments. (B) Fluorimetric measurement of fluorescence in MUG stained cell lysates. The results are reported as the rate of increase in fluorescence in arbitrary units. The values shown are representative of 3 independent assays and are the means and standard deviations obtained from stimulations performed in triplicate.

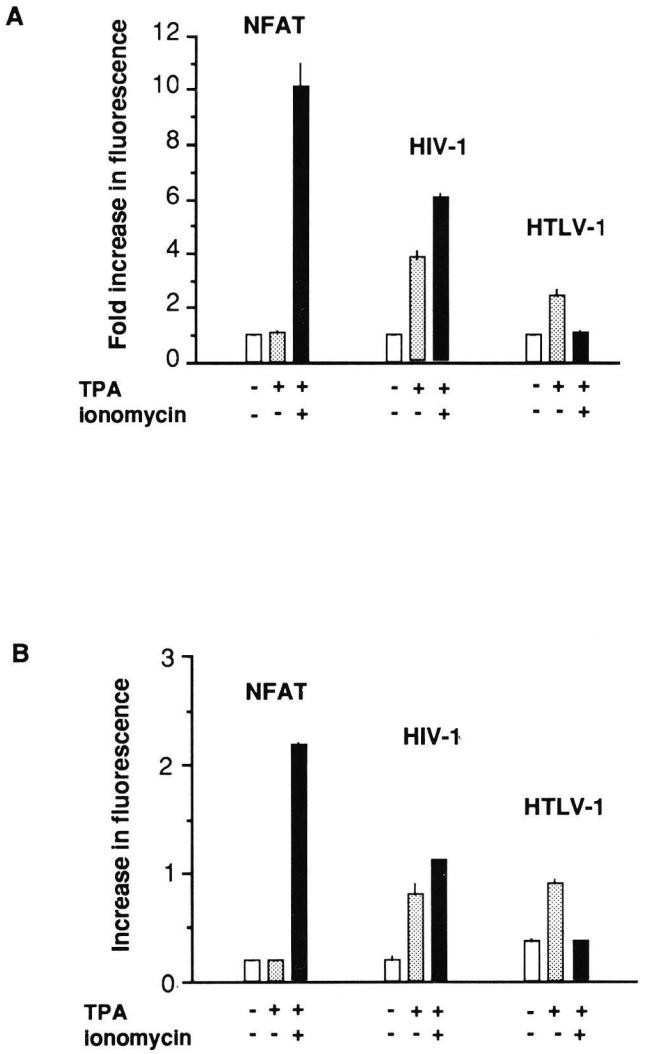

Figure 4. *LacZ expression in response to direct activation.*
Cells were cultured for 18 hours with TPA or TPA plus ionomycin and lacZ expression was measured (A) by flow cytometry of FDG stained cells as described in the legend to Figure 3A. Results are the mean and standard deviation of 4-6 independent experiments. (B) Fluorimetric measurement of fluorescence in MUG stained lysates. Results are representative of 3 independent assays and are the means and standard deviations obtained from triplicate stimulations.

ionomycin, higher levels of lacZ were produced and no delay in expression was observed. The HTLV-1 LTR produced maximal lacZ levels following 6 hours of TPA treatment, and expression was maintained throughout the entire sampling period. Exposure of HTLV-1 LTR expressing cells to antiCD3 mAb did not elicit a response from the LTR over the 24 hour sampling period. Cell cycle progression was monitored during time course stimulation experiments (Figure 5 A-F). All treatments resulted in an increase in the percentage of the cell population in G_2, S or M phases. These increases peaked between 12-24 hours post-stimulation. The percentage of cells entering G_2, S and M phases was highest following treatment with antiCD3 mAb.

DISCUSSION

Many studies have investigated the response of the HIV-1 LTR to cell surface and mitogenic triggering using transient reporter assays. These assays have a number of significant drawbacks, many of which are related to the disruption of normal physiological events in the cell caused by the traumatic methods by which foreign constructs are introduced into the cell. To study questions of proviral latency in human T-cells, we used the Jurkat T-cell model containing stably integrated LTRs of HTLV-1 or HIV-1 and compared their responses to cell surface signals to the IL-2 NF-AT enhancer. We have shown that the stably integrated HIV-1 LTR is activated via signals from the cell surface TCR/CD3 complex, as suggested by previous studies using transient transfections (93, 191, 293). However, optimal HIV-1 LTR activity was produced by direct activation of PKC and Ca^{2+} pathways by TPA and ionomycin, respectively. NF-AT-directed gene expression was absolutely dependent upon the activation of PKC and Ca^{2+} dependent pathways as previously reported (68). Expression from the HTLV-1 LTR did not occur in response to signals from the TCR/CD3 complex.

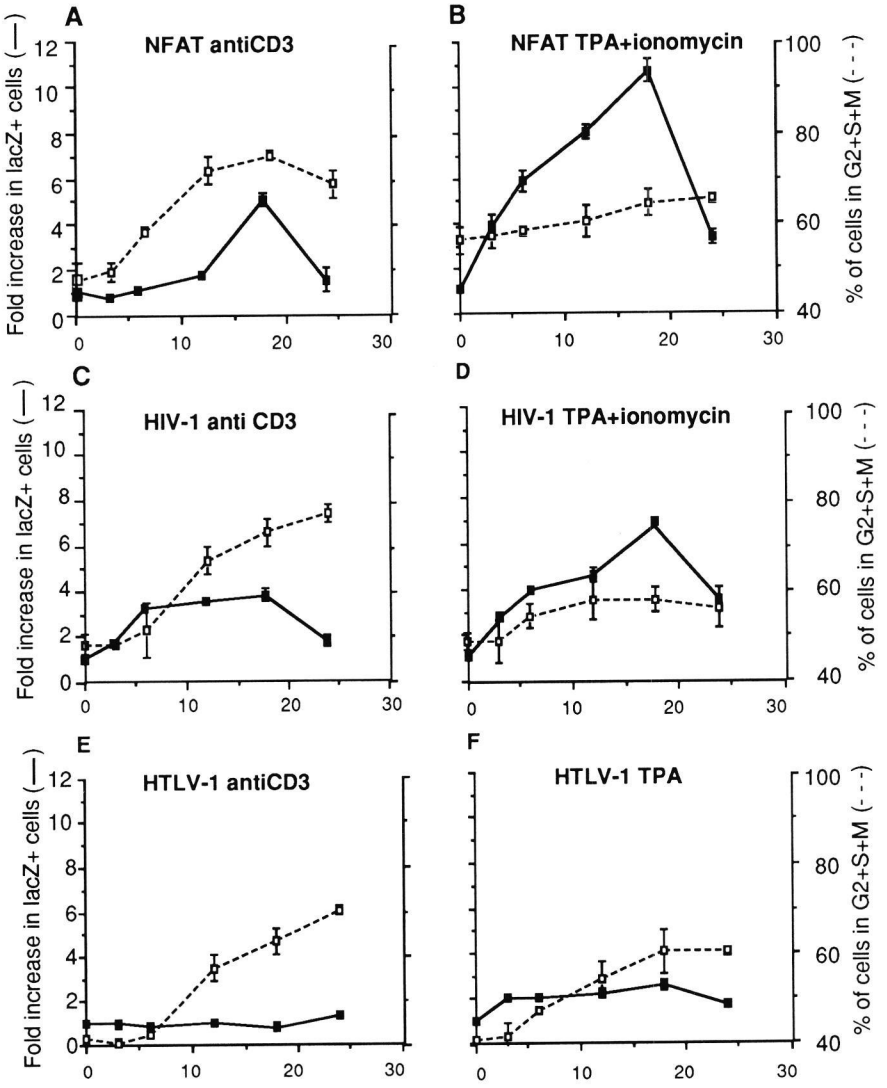

Figure 5. *Comparison of lacZ expression with cell cycle progression.*
LacZ expression (solid lines) and cell cycle progression (broken lines) were measured at 0, 3, 6, 12, 18 and 24 hours following stimulation with the indicated T-cell activating agents. The fold increase in lacZ expression is measured on the left axis. Cell cycle progression is measured on the right axis.

Triggering of the HIV-1 LTR using mAb to CD3 resulted in more immediate lacZ expression than was observed for the NF-AT enhancer in time course studies. In all time course experiments, lacZ expression preceded significant changes in cell cycle distribution with the exception of NF-ATlacZ cells treated with antiCD3 mAb. A dependence of expression upon cell cycle in this case is unlikely. The lag period associated with expression is more likely dependent upon achieving the required threshold level of free Ca^{2+} or Ca^{2+}-calmodulin dependent phosphatase activity for cytoplasmic NF-AT translocation to the nucleus (260). The more rapid response of the HIV-1 LTR to antiCD3 mAb may be explained by the ability of the HIV-1 LTR to respond to signals provided by PKC in the absence of elevated Ca^{2+}.

Stimulation via the CD28 pathway without concurrent TCR/CD3 stimulation does not induce T-cell proliferation (98, 159, 196) but can augment proliferation induced by suboptimal levels of mAbs that cross-link the TCR (159, 196) or CD2 receptor (160, 304). The pathway utilized by CD28 appears to be distinct from the phosphatidylinositol pathway activated by TCR engagement and in addition, delivers a signal which results in the stabilization of mRNAs of T_H1-specific lymphokines (128, 292). It has been reported that activation of T-cells via the CD28 receptor can increase the response of the HIV-1 LTR to antiCD3 mAb stimulation or to the combination of TPA plus PHA (293). Experiments by Gruters et al. (93) demonstrated that in Jurkat cells co-transfected with a HIV-1 LTR-CAT vector and a *tat* expression vector, stimulation with antiCD28 mAb could enhance LTR-directed expression. We demonstrate for the first time that an integrated HIV-1 LTR can respond to antiCD28 mAb in the absence of costimulatory factors such as HIV *tat* or mitogens. Verweij and coworkers (307) reported that stimulation of Jurkat cells via the CD28 receptor upregulated NFκB expression and could complement activation of a tandem repeat of the HIV-1 NFκB-like element by antiCD3 mAb. However it has also been demonstrated that mobilization of NFκB does not necessarily result in upregulation of HIV-1 expression (103). The lack of complementation of LTR-mediated lacZ expression by

antiCD3 and antiCD28 mAb in our experiments may reflect our use of the entire, intact LTR rather than the NFκB enhancer alone. Thus, our results suggest the presence of functional negative regulatory elements in the HIV-1 LTR or alternatively, the difference may be due to integration.

The response of the HTLV-1 LTR to PKC activation by TPA was inhibited by the elevation of the concentration of free Ca^{2+} by ionomycin. This result may have important implications as to the long latent nature of HTLV-1 infection and minimal pathologic effects in the majority of individuals. Furthermore, stimulation of HTLV-1 LTR-lacZ expressing cells via the CD3 or CD28 pathways did not induce lacZ expression. HTLV-1 has been described as a mitogenic agent in that it can activate human T-cells and does so independently of IL-2 production (81). How HTLV-1 expression in T-cells is initially triggered is not clear from our studies however, activation of Ca^{2+}-dependent pathways within an HTLV-1 infected cell may encourage the latency of the virus. Changes in the free Ca^{2+} availability per se may not be fundamental to this suppression as it may result from other Ca^{2+}-dependent T-cell activation events such as the activation of Ca^{2+}-calmodulin-dependent protein phosphatases.

These results suggest that a quiescent HIV-1 LTR can respond rapidly to T-cell activation signals. The ability of the LTR to respond to single signals (TPA, CD28 mAb) further suggests that HIV-1 may be responsive in aberrantly activated T-cells such as those which undergo anergy in the asymptomatic phase of infection. In contrast, the HTLV-1 LTR appears unresponsive to antigen-mediated T-cell activation pathways. Enhancer elements within the HTLV-1 LTR do not bear a strong resemblance to other promoter elements found in T-cell activation responsive genes. HTLV-1 may require another significant event, independent of T-cell activation, for its expression. The negative effect of calcium mobilization on HTLV-1 LTR-directed gene expression may play a central role in maintaining a latent-like state of the provirus during infection which might explain the lack of routinely detectable levels of HTLV-1 transcripts in infected cells, despite evidence of

random, non-clonal provirus integration.

In conclusion, studies of gene expression directed by an integrated LTR have provided novel information on the responses of two human retroviruses to T-cell activation signals and mechanisms which may influence proviral latency in T-cells. Such an approach allows a more accurate representation of the natural situation of the infected T-cell. FACS/FDG measurement provides for a rapid and reproducible detection of lacZ expression in viable, single cells. The preservation of cell viability in this assay permits multiparameter analyses using fluorescence measurement and the correlation of gene expression with other cell functions, including cell cycle analyses, expression of T-cell activation markers and the measurement of calcium ion mobilization. Assaying a reporter gene which has been stably integrated, instead of one which is transiently transfected, provides a more accurate assessment of the possible responses of provirus to normal immunological signals in T-cells.

Acknowledgements

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CHAPTER V

INHIBITION OF PHORBOL ESTER- AND *TAX*-MEDIATED *TRANS*-ACTIVATION OF THE HUMAN T-CELL LEUKEMIA VIRUS 1 LONG TERMINAL REPEAT BY IONOMYCIN

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SUMMARY

Human T-cells containing an integrated HTLV-1 LTR-lacZ reporter vector were used to study the role of calcium-dependent cellular activation pathways in LTR *trans*-activation. Treatment of the LTR-lacZ cells with the calcium ionophore ionomycin resulted in a reduced basal response of the LTR and reduced responses to TPA- and *tax*-mediated *trans*-activation. This effect was also observed for virus production by the HTLV-1 producing T-cell line MT-2. Experiments designed to determine the events underlying this inhibition revealed that the ionomycin-induced repression of the LTR was alleviated in all cases by cyclosporin A. Cyclosporin A was also effective in preventing the ionomycin-induced reduction in virus production in MT-2 cells. These results suggest a role for a calcium-related T-cell activation event in suppressing virus expression.

INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) infection is characterized by a long asymptomatic period in infected individuals. Infection is associated with adult T-cell leukemia/lymphoma (ATL) (106, 236, 323) and the inflammatory/degenerative neurological disorders tropical spastic paraparesis (TSP) and HTLV-1-associated myelopathy (HAM) (84, 229). ATL is a malignancy of helper T lymphocytes while HAM/TSP is a myelopathy which shares some of the characteristics of multiple sclerosis. Peripheral blood mononuclear cells of infected individuals undergo spontaneous proliferation *in vitro* (118, 119, 291) which may result from the activation of interleukin 2 (IL-2) and its receptor (IL-2R) by HTLV-1. The alteration of normal cellular function by

HTLV-1 has been proposed to be associated with the expression of viral proteins such as the *trans*-activator *tax* which can induce IL-2R expression (291). However, while HTLV-1 may induce transient activation of T-cells, there is no evidence to suggest that virus production is supported or enhanced in an activated cell.

Positive regulators of HTLV-1 LTR-directed gene expression include 12-O-tetradecanoylphorbol-13-acetate (TPA) (243) and *tax* (280, 29, 306, 243). The activation of HTLV-1 by *tax* is mediated via *tax*-responsive elements reiterated three times within the LTR (29, 280, 306). Transcriptional activation of cellular and other viral promoters by *tax* has been reported (4, 9, 72, 75, 164, 169, 173, 276). The response of the LTR to TPA-mediated activation is determined by a 60 bp sequence which overlaps with one *tax*-responsive element (243). The pathways by which *tax* and TPA *trans*-activate LTR-mediated gene expression are independent, demonstrated by the synergistic response of the LTR when both agents are used together (243).

Using a HeLa cell model, we have demonstrated that while the LTR is responsive to TPA, this response is significantly reduced by the calcium (Ca^{2+}) ionophore ionomycin (44). Ionomycin enhances the release of Ca^{2+} from intracellular stores and the influx of Ca^{2+} from the extracellular environment (39, 80, 82). Thus a T-cell activation associated pathway which enhances free Ca^{2+} concentration within the cell may present conditions which are unfavourable for virus expression. Studies of the influence of Ca^{2+}-mediated activation pathways on the HTLV-1 LTR have not been reported to our knowledge. In this report we have used Jurkat cells as a model for dissecting the Ca^{2+}-dependent events which are responsible for inhibition by ionomycin. Ca^{2+} regulation may be studied using inhibitors of specific Ca^{2+}-dependent events which occur following stimulation of T-cells by ionomycin. These inhibitors include EGTA and 1,2 bis (aminophenoxy) ethane N,N,N',N' tetraacetic acid (BAPTA/AM) which chelate extracellular and intracellular Ca^{2+}, respectively (5, 82, 69). The immunosuppressive drug cyclosporin A (CsA) inhibits Ca^{2+}-dependent signal transduction events essential for the expression of genes required during

T-cell activation (65, 70, 200, 244). In the experiments described here, we have used these inhibitors to dissect Ca^{2+}-dependent signalling events in Jurkat cells containing a stably integrated HTLV-1 LTR linked to the bacterial β-galactosidase (lacZ) gene. We report on the role of these Ca^{2+}-dependent events in activation of the LTR mediated by *tax* and TPA and on full virus production.

MATERIALS AND METHODS

Construction of vectors and stable transfections

The vector pHTLV-I LTR-lacZ-neo has been previously described (44). The LTR region of this vector spans -322 - +332 bp relative to the CAP site. pRSV*tax* is the HTLV-1 equivalent of the vector pBLV*tax* (50). The vector pHTLV-1 LTR-lacZneo was introduced into human Jurkat cells by electroporation. Jurkat cells ($2x10^6$) were suspended in 250 µl RPMI culture medium supplemented with 20% fetal calf serum. The cells were mixed with 10 µg of plasmid DNA and transferred to a 0.4 cm gap electroporation cuvette (Biorad) and electroporated (250V, 960 µF) using a Gene Pulser (Biorad) with capacitance extender. Cells were cultured for 2 days in RPMI supplemented with 20% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin before selection in 1 mg/ml G418 (Gibco-BRL). Cells were later cultured in Iscove's modification of Dulbecco's medium for cloning from single cells by limiting dilution.

Cell treatments

pHTLV-1 LTR-lacZ expressing Jurkat cells were transfected with 10 µg pRSV*tax* using a modified DEAE-dextran transfection procedure (93). Following 40 hours of culture, transfected and untransfected cells were split for treatment with ionomycin (2 µM; Sigma), TPA (10 ng/ml; Sigma) or TPA plus ionomycin, with and without the inhibitors

EGTA (2 mM), BAPTA/AM (5 μm; Sigma) or CsA (1 μg/ml; Sandoz). Culture medium contained 1 mM Ca^{2+}. To chelate extracellular calcium, cells were treated with EGTA for 5 minutes prior to the addition of TPA or ionomycin. For this treatment group EGTA was kept in the culture medium during the entire stimulation period. In the case of intracellular calcium chelation, cells were cultured in 5 μM BAPTA/AM for one hour and cells were then stimulated in the absence of BAPTA/AM. CsA was added to cells 10 minutes prior to the addition of TPA or ionomycin. Stimulation with TPA or ionomycin was 18 hours in duration.

β-galactosidase measurement

β-galactosidase was measured from the fluorescence produced following cleavage of the β-galactoside bond of 4-methyl umbelliferyl-β-D-galactoside (MUG, Molecular Probes). Cells were washed with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCL, 1 mM $MgSO_4$, pH 7.0) and lysed in Z buffer containing 0.1% Triton X-100. For fluorimeter measurements, 20 μl of 50 mM MUG was combined with 2 ml PBS and lysate of 2 x 10^5 cells (100 μl) in a quartz cuvette. Fluorescence at 376 nm was measured for one minute using 350 nm excitation with a luminescence spectrometer (LS50, Perkin Elmer). The rate of increase in fluorescence was calculated using linear regression analysis.

Measurement of HTLV-1 expression

HTLV-1 expression was detected as p24 antigen production using an enzyme immunoassay kit (HTLV-I, II Antigen Assay; Coulter). Equal numbers of MT-2 cells were cultured in the presence of increasing increments of ionomycin or ionomycin plus CsA for 72 hours. The supernatants were then used to measure p24 antigen, according to the manufacturers specifications. At the same time cell viability was determined, using trypan blue exclusion, to ensure that observed changes in virus production were not due to toxic effects of the reagents.

Measurement of intracellular calcium

Cells were prepared for intracellular Ca^{2+} measurement according to Vandenberghe and Ceuppens (302) with minor modifications. Briefly, cells (10^7/ml) were incubated 20 minutes under subdued light at room temperature in Hank's Balanced Salt Solution (HBSS) containing 20% Pluronic F-127 (Molecular Probes) in DMSO. An equal volume of HBSS containing 2 μM Fluo-3/AM (206) (Molecular Probes) was added to the cells and incubation was continued for one hour. Cells were then washed three times with HBSS and maintained on ice until measurement. Prior to loading cells with Fluo-3/AM, the cells were treated with calcium pathway inhibitors. BAPTA/AM was added to cells for one hour and then removed prior to staining with Fluo-3/AM. In the case of cyclosporin A and EGTA, these were added to the cells 10 minutes prior to staining with Fluo-3/AM and were present in solutions in all subsequent steps. The cells were transferred to 37^0 C for 10 minutes before measurement. Each treatment group was subdivided into four groups which received either no additions or ionomycin (2, 5 and 10 μM). Fluo-3/AM fluorescence (green) was measured at selected time points following the addition of ionomycin using a FACScan flow cytometer (Becton Dickinson). Excitation was from an argon laser at 488 nm. Emission at 530 nm was measured on a logarithmic scale. During the sampling period cells were maintained at 37^0 C.

RESULTS

Ionomycin is inhibitory to activation of the HTLV-1 LTR by TPA and tax

To investigate the requirements of cellular free Ca^{2+} in *trans*-activation of the HTLV-1 LTR, a Jurkat T-cell clone containing a stably integrated HTLV-1 LTR-lacZ gene was used. LTR-directed lacZ expression was measured by fluorimetry of cell lysates stained with the fluorogenic substrate MUG and is reported as the fold increase in lacZ expression

obtained in stimulated cells with respect to untreated cells. We have previously reported on the inhibitory effect of the Ca^{2+} ionophore ionomycin in the response of the HTLV-1 LTR to TPA in stably transfected HeLa cells (44). This inhibition was also produced in the Jurkat cell line. Figure 1 shows the effect of 2 μM ionomycin on basal LTR activity and on

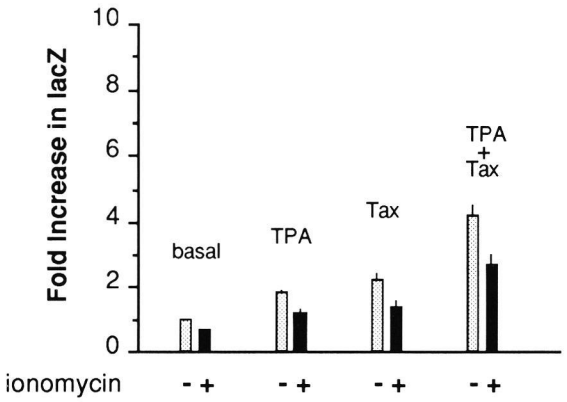

Figure 1. *Inhibition of LTR-mediated lacZ expression by ionomycin. pHTLV-1 LTR-lacZ expressing Jurkat cells were treated as indicated in the presence or absence of ionomycin (2μM). Fluorescence was measured in MUG stained lysates as described in Materials and Methods. The values shown are the means and standard deviations obtained from 4-6 independent experiments.*

activation mediated by TPA, *tax* or the combined treatment of *tax* plus TPA. The concentration of ionomycin used in these experiments is similar to that employed in other studies of gene expression during T-cell activation (68, 93, 200, 276, 300). In all cases ionomycin reduced LTR-directed gene expression by 20-25%.

The effect of calcium pathway inhibitors on intracellular Ca^{2+}

The effects of CsA and both chelators on Ca^{2+} mobilization were verified prior to activation studies by the measurement of intracellular Ca^{2+}. Figure 2A depicts the bimodal production of Ca^{2+} in the absence of inhibitors as detected by flow cytometric measurement of Fluo-3/AM stained cells. The initial release of Ca^{2+} from intracellular stores is evident by

1 minute following ionomycin treatment and is followed by a sustained plateau, representing the influx of Ca^{2+} from the extracellular environment. The initial increase in Ca^{2+} is reduced by BAPTA/AM treatment due to the chelation of intracellular Ca^{2+} (Figure 2B) and the sustained plateau is prevented by EGTA which chelates extracellular Ca^{2+} (Figure 2D). In the case of cells treated with CsA (Figure 2C), the bimodal release of Ca^{2+} was similar to that observed in the absence of CsA. The effectiveness of CsA was further tested using a Jurkat cell line expressing a trimeric NF-AT element linked to lacZ (68). The response of the NF-AT element to TPA plus ionomycin stimulation was efficiently blocked by CsA at 1 μg/ml (data not shown) as has been previously described (70, 276).

Ionomycin-mediated inhibition is recovered by CsA

HTLV-1 LTR activation was then investigated in cells treated with Ca^{2+} inhibitors in an attempt to identify the mechanism of inhibition by ionomycin. Cells were treated with BAPTA/AM (5μM) to chelate intracellular Ca^{2+}, EGTA (2mM) to chelate extracellular Ca^{2+} or with cyclosporin A (1μg/ml) to inhibit Ca^{2+}-sensitive T-cell signal transduction pathways. As experimental controls, basal LTR activity was tested in cells treated with Ca^{2+} inhibitors in the absence or presence of ionomycin. Basal lacZ expression in the absence of ionomycin was unaffected by chelation of intracellular Ca^{2+} by BAPTA/AM but showed a dependence upon extracellular Ca^{2+} availability (Figure 3A). CsA was able to slightly enhance basal LTR activity. In cells treated with ionomycin, basal LTR expression was decreased but this inhibition was not observed in the presence of ionomycin plus CsA. In fact, ionomycin and CsA were complementary resulting in a 45% increase in lacZ expression over basal levels.

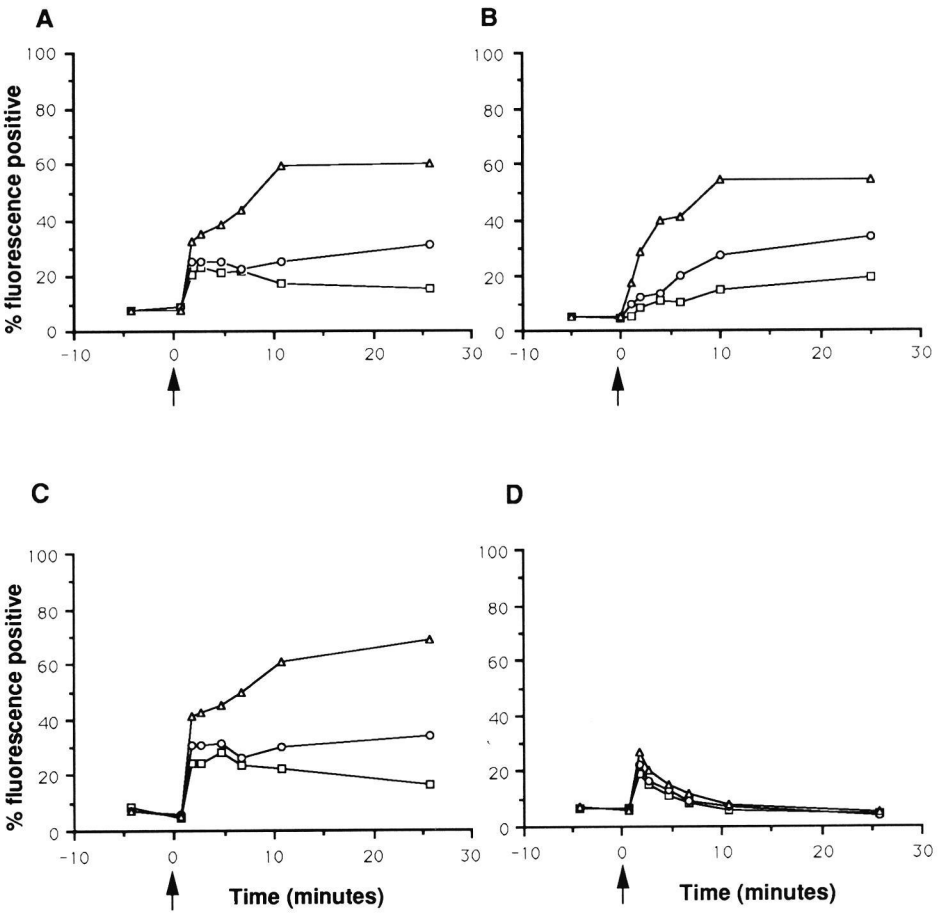

Figure 2. *Ionomycin-induced Ca^{2+} flux in Jurkat cells treated with pathway inhibitors. Prior to Fluo-3/AM staining the cells were treated with A: medium alone; B: BAPTA/AM 5μM; C: CsA 1 μg/ml; D: EGTA 2mM. To measure Ca^{2+} mobilization, stained cells were treated with ionomycin at 2 (square); 5 (circle); and 10 (triangle) μM. The point of addition of ionomycin is indicated by an arrow. Each point represents the percent of the population positive for Fluo-3/AM fluorescence. A representative of 3 independently performed experiments is shown.*

The response of the LTR to TPA treatment was shown to be dependent upon intracellular Ca^{2+} levels and the availability of extracellular Ca^{2+}, as both BAPTA/AM and EGTA treatments reduced the lacZ activity to near basal levels in TPA-treated cells (Figure 3B). TPA-mediated expression was not affected by treatment with CsA. Ionomycin suppressed TPA-mediated responses and this decrease in gene expression was not alleviated when intracellular and extracellular Ca^{2+} were chelated by BAPTA/AM and EGTA, respectively. However, the suppression induced by ionomycin was overcome in the presence of CsA and TPA-responsiveness was restored to levels slightly higher than observed in the absence of ionomycin.

The requirements of Ca^{2+} in *tax*-mediated *trans*-activation of the LTR were investigated following transient transfection of the pHTLV-1 LTR lacZ expressing cells with pRSV*tax* (Figure 3C). The response of the LTR to *tax* was not adversely affected by chelation of intracellular Ca^{2+} by BAPTA/AM or extracellular Ca^{2+} by EGTA. Ionomycin treatment reduced the response of the LTR to *tax*-mediated *trans*-activation and this repressive effect was alleviated by chelation of intracellular Ca^{2+} by BAPTA/AM and to a greater degree by both EGTA and CsA treatment. The chelation of Ca^{2+} mildly reduced the synergistic response of the LTR to *tax* plus TPA (Figure 3D). This response was slightly enhanced by CsA. The response of the LTR to the combined treatment of *tax* plus TPA was again inhibited by ionomycin and in this case the inhibition was recovered by CsA. Surprisingly, the recovery resulted in much higher levels of lacZ expression than observed for *tax* plus TPA treatment in the absence of ionomycin, suggesting again that CsA and ionomycin have complementary effects upon LTR-directed gene expression.

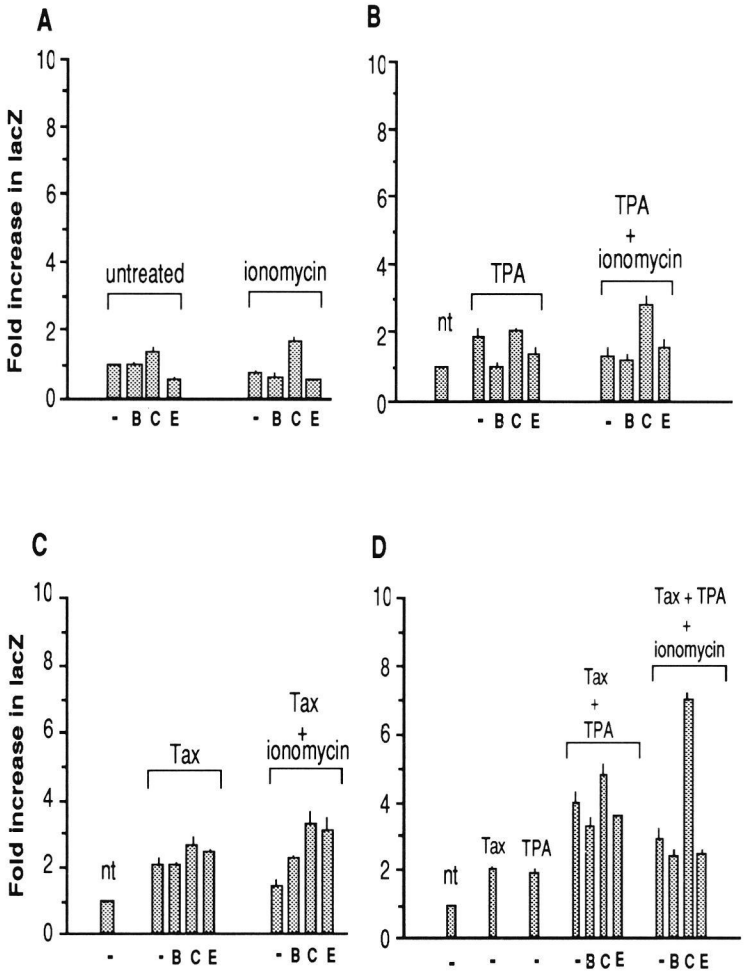

Figure 3. *Investigation of ionomycin-induced inhibition using calcium pathway inhibitors. Fluorescence was measured by fluorimetry of lysates of untransfected or pRSVtax-transfected HTLV-1 LTR-lacZ cells. Cells were pre-stimulated with medium alone (-); BAPTA/AM 5μM (B); CsA 1μg/ml (C); or EGTA 2mM (E) prior to treatment with TPA or ionomycin. The results represent the means and standard deviations of 3-5 independent experiments.*

Ionomycin inhibits virus expression by MT-2 cells

To further characterize the role of Ca^{2+} in the suppression of HTLV-1 expression, we examined the effects of ionomycin on full virus expression in MT-2 T-cells. Virus production was also measured and compared for untreated MT-2 cells and CsA treated cells exposed to increasing increments of ionomycin. Table 1 shows the effect of these reagents on virus expression measured by p24 production. Decreases in virus expression were evident at 10 μM of ionomycin. Although toxic effects of ionomycin were sometimes observed at 5 and 10 μM, the reduction in virus expression in these experiments did not correlate with reduced numbers of viable cells. Increased virus expression was not observed in cells treated with ionomycin plus CsA. However, in the presence of CsA virus expression was not reduced by ionomycin at 10 μM.

Table 1. *Influence of Ionomycin and CsA on HTLV-1 production in MT-2 cells*

Ionomycin (μM)	p^{24} (μg/L) per 10^6 viable cells[a]
0	104
2	116
5	92
10	55

Ionomycin (μM)	CsA (1μg/ml)	p^{24} (μg/L) per 10^6 viable cells
0	-	65
0	+	61
2	+	60
5	+	72
10	+	78

[a] *Virus production was detected as p^{24} production using a HTLV-I/II specific antigen capture kit. Viable cell number was calculated using trypan blue exclusion prior to p24 measurement.*

DISCUSSION

In this study, we have demonstrated that ionomycin can reduce basal LTR responses in addition to activation via TPA and/or *tax*. The mechanism underlying this inhibition was investigated using Ca^{2+} chelators. In addition, the immunosuppressive drug CsA was employed to block a Ca^{2+}-sensitive T-cell signal transduction pathway (94, 139, 170, 200). Interaction of CsA with calcineurin, a Ca^{2+}/calmodulin-regulated phosphatase, has recently been shown to be the mechanism controlling cyclosporin A-induced immunosuppression. Cyclosporin A blocks transcription of the IL-2 gene by inhibiting the phosphatase activity of calcineurin which is required for the binding activity of the transcription factors NF-AT and NF-IL2A. The requirement for calcineurin in the activation of NF-AT and NF-IL2A in Jurkat cells has been demonstrated (40).

Chelation of extracellular versus intracellular calcium by the agents EGTA and BAPTA/AM, respectively, revealed that indeed calcium ions are necessary for the response of the LTR to TPA-mediated activation. This result is not surpising as free Ca^{2+} is a co-requirement for translocation of protein kinase C to the cell membrane (20). *Trans*-activation mediated by *tax* did not demonstrate a dependence on Ca^{2+} mobilization as chelation of Ca^{2+} did not affect levels of *tax*-induced lacZ expression. Therefore *tax* does not appear to require Ca^{2+} as a co-factor in upregulating the activity of LTR binding proteins. However, chelation of intracellular and extracellular Ca^{2+} did relieve the inhibition of *tax*-mediated *trans*-activation imposed by ionomycin. This suggests that binding proteins elicited by *tax* may be down-regulated or perhaps have a reduced affinity for the LTR in the presence of elevated levels of free Ca^{2+}.

In our experiments, CsA was upregulatory to HTLV-1 LTR-directed gene expression. Although ionomycin inhibited TPA- and *tax*-mediated expression from the LTR, ionomycin and CsA used in concert enhanced responses to TPA and *tax* above levels obtained in the absence of ionomycin. These results suggest that an increase in Ca^{2+} may

have two effects. One effect results in the inhibition of HTLV-1 LTR *trans*-activation and this inhibition is in turn recovered by CsA. The second effect appears to mediate an increase in *trans*-activation, but this is only detected when the negative effect is blocked by CsA. Several scenarios can be suggested to explain this result. Ionomycin may transmit a negative signal which could result in either a reduced affinity of transcription factors for the LTR or reduced expression of these molecules overall. This is not likely caused solely by elevated intracellular Ca^{2+} levels since chelation of Ca^{2+} did not alleviate ionomycin-induced inhibition in all cases. In addition, the observation that CsA does not have a significant effect upon the mobilization of Ca^{2+} by ionomycin within the cell, as measured in Fluo-3/AM labelled cells, suggests that the mode of action of the ionomycin-induced inhibiton is likely the result of a CsA suppressible T-cell activation event(s). Protein-bound CsA, which is unable to enter the cell, can mediate an inhibition of T-cell activation in a similar way as can uncomplexed CsA (25). If CsA is able to act at the cell surface, then this activity may stimulate an alternate activation pathway to which the HTLV-1 LTR is responsive. Alternatively, CsA may prevent the occurrence of a Ca^{2+}-dependent T-cell activation pathway, and in this way block signals which are detrimental to HTLV-1 expression. The complementation of *trans*-activation by ionomycin plus CsA may result from suppression of an inhibitory pathway by CsA combined with a higher affinity of factors for the LTR due to enhanced Ca^{2+} levels.

Ionomycin has been shown to reduce the response of the IL-2 promoter to phorbol ester (300) and this reduction was recovered by CsA. Phorbol ester activation of the IL-2 gene is mediated by the AP-1 binding element of the promoter. However, while the authors were able to demonstrate down-regulation at the level of gene expression, no differences in Jun binding to the AP-1 element were evident in the presence of ionomycin. We are currently attempting to identify the LTR sequences responsible for ionomycin inhibition and CsA recovery. As ionomycin affects basal, *tax* and TPA-induced activation, the site(s) involved may be repeated throughout the LTR, including the U5 region which was recently

identified to house sequences controlling basal expression (137).

Although ionomycin reduced virus production by MT-2 cells, we were unable to demonstrate that the combination of CsA plus ionomycin had an upregulatory role on full virus expression. However, the ionomycin-mediated reduction in virus expression did not occur in the presence of CsA. As MT-2 cells are strong producers of HTLV-1, the cells may not be able to support further increases in virus production. We have observed additionally that treatment of MT-2 cells with TPA does not result in detectable increases in virus production (data not shown). However, the possibility exists that cellular signals may act to suppress HTLV-1 expression. This is supported by the low expression of the virus in infected cells.

HTLV-1 has been demonstrated to provide a mitogenic signal to T-cells and to activate T-cells in the absence of accessory cells (57). The mitogenic activity of HTLV-1 is restricted to infected T-cells and requires cell-to-cell contact (145). As infected cells express high levels of IL-2 receptor, infection thus affords to the cell an activated state. Given that our results suggest a Ca^{2+}-related T-cell activation event can be inhibitory to LTR-mediated gene expression, we propose that T-cell activation may be one of several factors which play an important role in sustaining relative viral latency in T-cells of infected individuals.

Acknowledgements

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CHAPTER VI

ANTI-APO-1 MEDIATED APOPTOSIS IS INHIBITED IN T-CELL LINES EXPRESSING HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 *TAX* PROTEIN

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submitted for publication

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SUMMARY

Cell surface mediated apoptosis was investigated in tumorigenic T-cell lines using the monoclonal antibody anti-APO-1. All cell lines expressed APO-1 on the cell surface and were susceptible to anti-APO-1 induced apoptosis. However, the degree of susceptibility to apoptosis was variable. Cells bearing inactive provirus or cells actively producing HTLV-1 or HTLV-1 *tax* protein showed higher resistance to anti-APO-1 induced apoptosis, despite the expression of high levels of cell surface APO-1. Cell-free supernatant of the virus-expressing cell line MT-2 and the *tax*-expressing cell line C8166 transferred increased resistance to anti-APO-1 to susceptible Jurkat cells. This resistance was not due to the intact virus as culture of Jurkat cells with heat-inactivated supernatant of MT-2 cells successfully protected the Jurkat cells against anti-APO-1-induced apoptosis. Jurkat cells transfected with an HTLV-1 *tax* expressing vector, or treated with soluble *tax* protein, were also less susceptible to anti-APO-1 induced cell death. Anti-APO-1-induced apoptosis was found to be inhibited in Jurkat cells by zinc sulphate, EGTA or by direct activation of protein kinase C (PKC) by 12-O-tetradecanoylphorbol-13-acetate (TPA). Treatment of MT-2 cells with staurosporine, an inhibitor of PKC resulted in an enhanced susceptibility of these cells to anti-APO-1. These results suggest that the resistance to anti-APO-1-induced apoptosis by HTLV-1/*tax* requires the integrity of the PKC activation pathway. Resistance to apoptosis by cells actively infected with HTLV-1 may be an important step towards HTLV-1-induced leukemogenesis.

INTRODUCTION

Programmed cell death, or apoptosis, is a natural process of cell elimination. During T cell ontogeny in the thymus apoptosis is required for the elimination of self-reactive thymocytes (180, 212). Activation-induced or T-cell receptor (TCR)-mediated apoptosis has been demonstrated to occur in T-cell hybridomas and thymocytes (201, 225, 278) and in mature CD4 and CD8 T-cells of HIV-1 infected individuals (8, 12, 88, 92, 204). Cell death by apoptotic pathways is the result of the activation of a cellular calcium-dependent endonuclease which cleaves chromosomal DNA at nucleosomal spacer regions to generate 180-200 base pair fragments (58, 320).

In vitro, tumorigenic T-cell lines or mature T-cells of healthy individuals are not susceptible to calcium ionophore- or TCR-induced cell death. Apoptosis can be induced following activation of mature T lymphocytes only under specific conditions which may result from comprimization of normal activation pathways (122, 216, 253). The monoclonal antibody anti-APO-1 recognizes the cell surface antigen APO-1, a member of the nerve growth/TNF receptor super family (47) which is homologous to the Fas lymphocyte surface antigen (117, 226). Anti-APO-1 has been demonstrated to be a powerful inducer of apoptosis and the administration of small amounts of APO-1 (1 ng/ml) to some cell types initiates rapid, extensive cell death (51, 296). Anti-APO-1 used in the treatment of murine tumours has been shown to cause a regression of tumor volume (296).

HTLV-1 is a human retrovirus which has been identified as the cause of the mature T-cell cancer adult T-cell leukemia/lymphoma (ATL) (106, 236, 323). Infection of T-cells with HTLV-1 is associated with the ability of these cells to proliferate in an interleukin-2 (IL-2) independent manner (54). This characteristic may be the result of the activation of the IL-2 receptor (IL-2R) by the viral-encoded *tax* protein. *Trans*-activation by *tax* has been demonstrated for the IL-2R gene (46, 164, 227) as well as many other cellular genes including *c-fos* (75), the human vimentin gene (169), HIV-1 (9, 276), TGF-α (4), TNF-β

and immunoglobin κ genes (173) and the human globin genes (72). Another characteristic of HTLV-1 transformed T-cells is the expression to high levels of the APO-1 cell-surface receptor (48). However, while ATL cells have been shown *ex vivo* to express high levels of APO-1, these cells may be intrinsically resistant to anti-APO-1-induced cell death (47). The mechanisms controlling the resistance to anti-APO-1 do not appear to involve IL-2 expression (48). Some viruses effect a dysregulation of normal cellular suicide programs to support viral integration and replication. Apoptosis is inhibited in adenovirus-infected B-cells by the expression of the E1B protein (245). Epstein Barr and Sindbis viruses inhibit cell suicide by upregulation of the *bcl-2* oncogene (104, 165). We have used mature tumorigenic T-cell lines to explore our hypothesis that expression of the HTLV-1 *tax* protein may play a key role in the resistance of cells actively infected with HTLV-1 to apoptosis.

MATERIALS AND METHODS

Cell lines and transfections

Jurkat T-cells were cultured in Iscove's Modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS) and streptomycin (100 μg/ml) plus penicillin (100 U/ml). MT-2, MT-4, Hut78 and C8166 cells were cultured in RPMI 1640 with the same supplements. For infection experiments using Jurkat cells, cells were cultured in RPMI for one week prior to infection. Jurkat cells cultured in Iscove's medium were transfected with pRSV*tax* (provided by David Derse; Laboratory of Viral Carcinogenesis Frederick Cancer Research Facility, Frederick, Maryland) or control vector pSV40*tat* (provided by Ben Berkhout; Human Retrovirus Laboratory, Academic Medical Center, Amsterdam) using a modified DEAE dextran procedure (93). Cells were then cultured for 48 hours prior to exposure to anti-APO-1 monoclonal antibody (mAb).

Reagents and cell treatments

The effective concentration of anti-APO-1 mAb (IgG3, κ) and time of incubation required to induce apoptosis in Jurkat cells were determined empirically. Apoptosis was induced in Jurkat cells at 1 ng/ml within 1 hour of culture with anti-APO-1 (data not shown). In subsequent experiments, apoptosis was measured after a 2 hour incubation with anti-APO-1 mAb using a range of concentrations (1-100 ng/ml). Soluble *tax* (172, 195) was provided by John Brady (Laboratory of Molecular Virology, National Cancer Institute, Bethesda MD). Cells were treated with soluble *tax* for 4 hours to allow the uptake of the protein by the cells prior to treatment with anti-APO-1 mAb. In experiments examining the inhibition of anti-APO-1-induced apoptosis, the cells were exposed to staurosporine (1 μM), cyclosporin A (1 μg/ml), 12-O-tetradecanoyl phorbol-13-acetate (TPA; 25 ng/ml) or TPA in combination with ionomycin (2 μM) for 18 hours prior to exposure to anti-APO-1. In experiments utilizing mAb-mediated activation, cells were cultured with immobilized antiCD3 (CLB-T3/3) or soluble antiCD28 (CLB-CD28/1) at 1 μg/ml. AntiCD3 and antiCD28 mAbs were kindly provided by Rene van Lier (Central Laboratory of the Netherlands Red Cross Transfusion Service, Amsterdam). Chelation of intracellular calcium (Ca^{2+}) was achieved by culturing cells with BAPTA/AM (5 μM) for one hour. The cells were then further treated with anti-APO-1 in the absence of BAPTA/AM. For chelation of extracellular Ca^{2+}, cells were cultured with EGTA (2mM) for 5 minutes prior to anti-APO-1 treatment and EGTA was kept in the medium during the anti-APO-1 culture period.

Measurement of cell surface expression of APO-1

Cells were incubated with anti-APO-1 or non-binding anti-human Leu16 (antiCD20) at a concentration of 70μg/ml in 100 μl phosphate buffered saline (PBS) containing 1% BSA for 30 minutes at 4^0 C. Following washing, 30 μl of a 1:80 dilution of FITC-labeled goat anti-mouse IgG (in 1% BSA, 0.02% sodium azide and 20% of 1:1 goat:monkey

serum) was added to the cell pellet and cells were then incubated at 4^0 C for a further 30 minutes. The cells were then washed with PBS and fixed in 1% formaldehyde in PBS. FITC fluorescence was detected using a FACScan flow cytometer (Becton Dickinson).

In situ nick translation

DNA fragmentation in cells was measured by *in situ* nick translation (126, 188, 204) using cells (1 x 10^6) previously fixed succesively in 1% formaldehyde and 70% ethanol. Cells were washed with nick buffer (5 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.8) and resuspended in 7 µl nick buffer plus 3 µl of a mixture containing 33 µM unlabelled nucleotides (dATP, dCTP, dGTP), 27.5 µM Biotin-labelled dUTP, 0.3 U DNA polymerase prepared in nick buffer. The reaction mixtures were incubated for 90 minutes at 15^0 C. Following washing, cells were then suspended in 40 µl of a mixture containing 2.5 µg/ml Avidin-FITC, 20 µg/ml DNase-free RNase, 0.1% Triton X-100, 4 X SSC, 5% nonfat milk (prepared in 4 x SSC) and incubated at room temperature 30 minutes. The cells were then counterstained in PBS containing 0.1% Triton X-100 and 5 µg/ml propidium iodide. Apoptotic cells were detected by the increase in FITC specific fluorescence (green) over control untreated cells using a FACScan flow cytometer.

Gel electrophoresis of cellular DNA

DNA was generally prepared from 5 x 10^5 cells. Following overnight incubation of the cells at 37^0 C in lysis buffer (PBS containing 1% SDS plus 200 µg/ml proteinase K), the DNA was extracted with phenol and precipitated with 0.3M sodium acetate and 2 volumes ethanol. The DNA was resuspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and of this volume 5 µl was loaded per well of a 1% agarose gel prepared in TBE buffer (89 mM Tris-HCl, 89 mM boric acid; 1 mM EDTA) containing ethidium bromide. Gels were photographed under UV illumination.

Infection of cells with HTLV-1

As targets, Jurkat cells were cultured in the supernatant of C8166 or Hut78 cells or supernatant of the virus-producing cells MT-2 and MT-4 for 48 hours. Prior to use, supernatant was passed through a 0.22 micron filter. Supernatants of HTLV-1 associated cells were tested for virus antigen using a Coulter HTLV I/II antigen capture kit. Inactivation of HTLV-1 in supernatant was performed by incubating the supernatant at 56^0 C for 30 minutes.

RESULTS

HTLV-1 infected and uninfected cell lines show variable susceptibility to anti-APO-1-induced apoptosis

We studied the expression of APO-1 by a panel of immortalized cell lines using FACS analysis of FITC-labelled anti-APO-1. While all cell lines showed high expression of APO-1, only Jurkat cells showed enhanced susceptibility to anti-APO-1 mAb, as measured by *in situ* nick translation (ISNT) of fragmented DNA. Apoptosis was induced in Jurkat cells at 1 ng/ml while the other cells required at least 10-fold higher levels of anti-APO-1. In Figure 1A, data comparing two methods for the detection of apoptosis in Jurkat and MT-2 cells are shown. The susceptibility of Jurkat cells to anti-APO-1 was determined by gel electrophoresis to detect the DNA ladder which is characteristic of apoptosis. Fragmentation is reduced in MT-2 cells. The percentage of apoptotic cells as determined by ISNT is indicated below the Figure. The results shown in Table 1 are a representative of 3 independent experiments.

Table 1. *Induction of apoptosis by anti-APO-1 in human T-cell lines*

Cell type	APO-1 expression: % population	APO-1 expression: Relative fluorescence intensity (APO-1/CD20)	% Apoptotic Cells[a] (ng/ml anti-APO-1): 0	1	10	100	HTLV-1 p24 ng/L[b]
Jurkat	78	6.5	22.3	47.5	75.6	97.8	0
C8166	84	4.1	17.3	23.8	52.8	46.1	0
Hut78	98	8.5	21.5	15.9	33.7	66.0	0
MT-2	83	4.1	22.8	21.0	26.1	72.7	347
MT-4	98	11.7	10.8	7.9	12.7	16.3	10

[a] *Percent apoptosis measured using in situ nick translation.*
[b] *Virus production was measured by detection of p24 antigen in supernatant of 1 x 10^6 cells /ml.*

Transfer of protection against anti-APO-1 to Jurkat cells

To determine whether cells resistant to anti-APO-1 could transfer this protection to susceptible cells, Jurkat cells were cultured in cell-free supernatant of MT-2, MT-4, C8166 and Hut78 cells. MT-2 cells produce high levels of HTLV-1 while MT-4 cells, although bearing the HTLV-1 genome, express minimal amounts of virus. C8166 bear but do not express the HTLV-1 genome. However, C8166 cells express high levels of the HTLV-1 *trans*-activating protein *tax*. In Table 2 the effect of 48 hour culture of Jurkat cells with the supernatants on subsequent anti-APO-1-induced apoptosis is shown. Supernatants of virus-associated cell lines were also heat-treated to inactivate HTLV-1. DNA fragmentation in Jurkat cells cultured in supernatant of the same cell type is evident at 1 ng/ml anti-APO-1 as measured by ISNT. Jurkat cells were protected from anti-APO-1 at 1 and 10 ng/ml by culturing in supernatants of MT-2 and C8166 cells. Heat inactivation of the virus did not affect the transfer of protection. The non-HTLV-1 producing cell line Hut78 and the HTLV-1 positive but non-expressing cell line MT-4 were not effective in conferring protection. In Figure 1B the transfer of resistance by MT-2 supernatant is shown by gel electrophoresis.

DNA fragmentation evident in Jurkat cells at 1-100 ng/ml of anti-APO-1 is inhibited at the same levels of anti-APO-1 in cells cultured in active MT-2 supernatant. The percentage of apoptotic cells as determined by ISNT is indicated below the Figure.

Table 2. *Transfer of protection from apoptosis to Jurkat cells by HTLV-associated cell supernatants*

Cell Source of Supernatant	Increase in % apoptosis[a] over untreated cells (anti-APO-1 ng/ml) 1	10
Jurkat	45.4	78.0
MT2	1.2	18.7
MT2 inactive	0	5.7
MT4	3.4	65.5
MT4 inactive	0	39.9
C8166	8.5	15.5
Hut78	25.6	57.2

[a] *DNA fragmentation in cells measured by in situ nick translation. The results are representative of 2-3 independent experiments.*

Protection from anti-APO-1-induced apoptosis by HTLV-1 tax protein

The ability of a *tax*-expressing cell line to transfer resistance to apoptosis was investigated further using Jurkat cells transfected with the *tax*-expressing vector pRSV*tax* and by cultivation of cells with soluble *tax* protein. As shown in Table 3, following transfection or culture with soluble *tax*, the level of apoptotic cells detected by ISNT is reduced as compared to cells treated with anti-APO-1 in the absence of *tax*. The protective influence of *tax* was eliminated in the presence of anti-*tax* polyclonal antibody. Transfection with the HIV-*tat*-expressing vector SV40*tat* did not inhibit anti-APO-1-induced apoptosis in Jurkat cells.

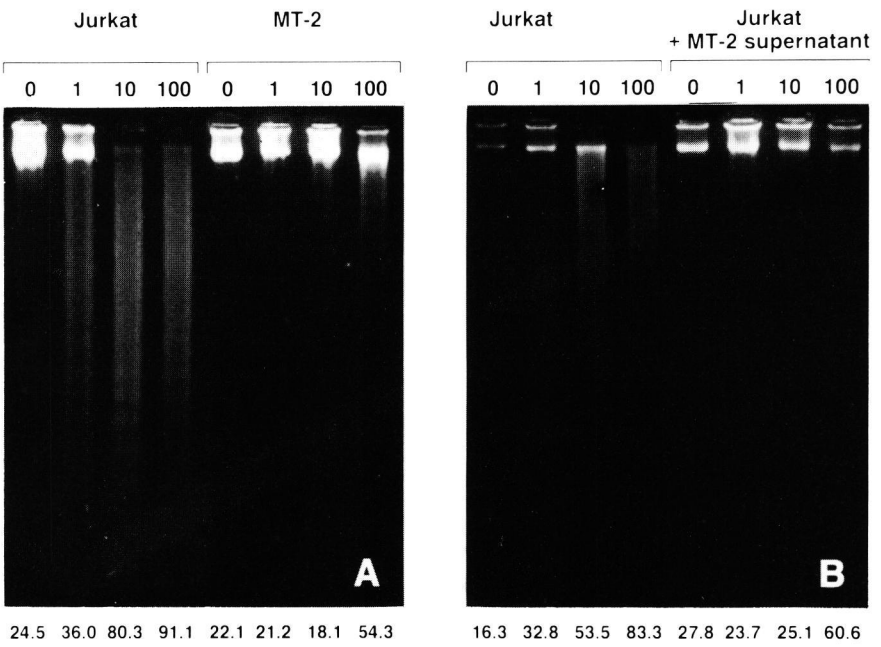

Figure 1. *Anti-APO-1-induced apoptosis in mature T-cell lines.*
(A) DNA of Jurkat and MT-2 cells treated with anti-APO-1 at 0, 1, 10 and 100 ng/ml. (B) DNA of Jurkat cells treated with anti-APO-1 and Jurkat cells cultured with supernatant of MT-2 cells for 48 hours prior to anti-APO-1 treatment. Numbers below each lane indicate the percentage of apoptotic cells as determined by in situ nick translation.

Pathways inhibitory to anti-APO-1

To understand the pathways utilized by anti-APO-1 in inducing apoptosis, a panel of reported inhibitors of apoptosis were tested. Jurkat cells were treated with these reagents as described in the Materials and Methods and were then subsequently cultured with anti-APO-1 at 10 ng/ml. Table 4 shows the ISNT results of a representative experiment of 3-4 independent experiments. Activation by anti-CD3 or anti-CD28 mAbs did not prevent or reduce anti-APO-1-induced apoptosis. Chelation of intracellular Ca^{2+} by BAPTA/AM was also ineffective as was the immunosuppressive drug cyclosporin A. Apoptosis was reduced to near basal levels by direct activation of PKC by the phorbol ester TPA and the

combination of TPA plus ionomycin. A similar reduction was also conferred by zinc sulphate which inhibits endonuclease activity and by chelation of extracellular Ca^{2+} by EGTA.

Table 3. *Apoptosis is reduced in cells expressing HTLV-1 tax* [a]

	Transient Transfection					
	untransfected		pRSV*tax*		pSV40*tat*	
Anti-APO-1 10 ng/ml	-	+	-	+	-	+
Experiment 1	21.7	91.8	16.3	54.7	13.7	80.7
Experiment 2	13.1	95.4	15.3	41.0	13.5	97.8

			Soluble *tax* (1 µg/ml)	
				Antitax 2 µg/ml
Anti-APO-1 10 ng/ml	-	+	+	+
Experiment 1	16.3	91.1	32.9	nd
Experiment 2	17.7	97.6	43.5	nd
Experiment 3	14.1	90.7	37.5	nd
Experiment 4	27.9	95.4	43.1	91.4
Experiment 5	15.6	95.6	43.5	91.4

[a] *% apoptotic cells measured by in situ nick translation*

MT-2 cells can be primed for anti-APO-1-induced apoptosis

We tested the effect of PKC inhibition on anti-APO-1-induced apoptosis in MT-2 cells. In contrast to the results of Traganos et al. (295) who reported that the protein kinase C inhibitor, H7, induced apoptosis in lymphocytes and Molt-4 cells, we found that staurosporine induced a generalized DNA degradation in Jurkat cells which was detected by ISNT. However, this degradation did not show the DNA ladder characteristic to apoptosis (data not shown). While MT-2 cells were more resistant than Jurkat cells to staurosporine-induced DNA degradation, pre-treatment of MT-2 cells with staurosporine resulted in an

enhanced sensitivity to anti-APO-1 at lower levels of the mAb. This can also be seen in Figure 2. As shown in lane 4, the effect of 10 ng/ml anti-APO-1 had minimal effects on the DNA of MT-2 cells. However, following culture with 1.0 μM staurosporine, the cells were more sensitive to the effects of anti-APO-1 at 10 ng/ml as is shown by the enhanced level of DNA degradation and the characteristic ladder appearance of the DNA (lane 8).

Table 4. *Effect of T-cell activating agents and apoptosis inhibitors on anti-APO-1 treated Jurkat cells*

	% Apoptotic Cells [a]	
Cell treatments	-	+[b]
Medium	14.9	89.0
Anti-CD3 mAb	12.5	82.9
Anti-CD28 mAb	13.0	86.5
TPA	15.8	27.1
TPA + ionomycin	22.4	26.6
BAPTA/AM	32.9	92.2
Cyclosporin A	18.3	88.8
EGTA	18.4	30.5
Zinc sulphate	15.2	19.1

[a] *% apoptotic cells measured by in situ nick translation*
[b] *- no anti-APO-1; + anti-APO-1 10 ng/ml*
The results shown are a representative of 3-4 independently performed experiments

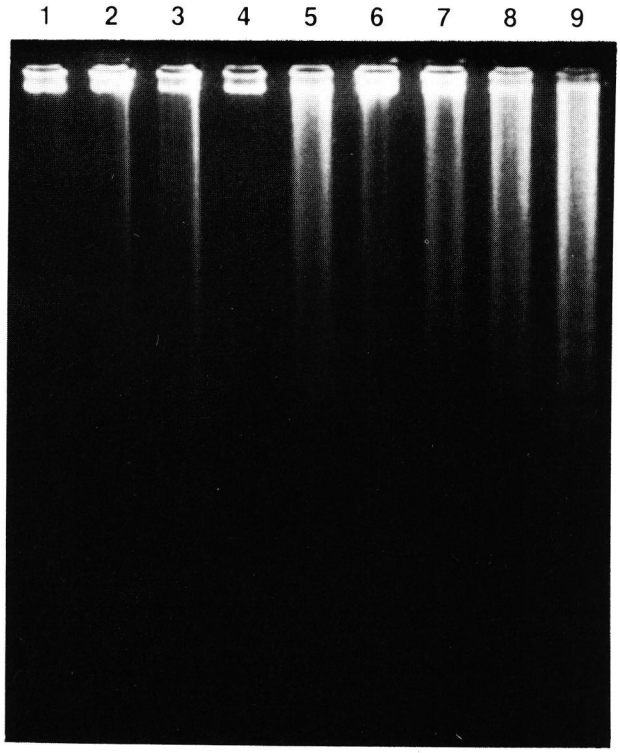

Figure 2. *MT2 cells can be primed for anti-APO-1-induced apoptosis.*
DNA was purified from MT-2 cells before and after anti-APO-1 treatment following 18 hours of culture with staurosporine. Lane 1, no treatment; lane 2, staurosporine 0.1 μM; lane 3, staurosporine 1.0 μM; lane 4, anti-APO-1 10 ng/ml; lane 5, anti-APO-1 100 ng/ml; lane 6, staurosporine 0.1 μM plus anti-APO-1 10 ng/ml; lane 7, staurosporine 0.1 μM plus anti-APO-1 100 ng/ml; lane 8, staurosporine 1.0 μM plus anti-APO-1 10 ng/ml; lane 9, staurosporine 1.0 μM plus anti-APO-1 100 ng/ml.

DISCUSSION

The level of induction of apoptosis induced in a panel of human T cell lines via the APO-1 cell surface protein was found to be highly variable despite the comparably uniform expression of APO-1. Human Jurkat cells, which phenotypically resemble resting T-cells,

were found to be most susceptible to anti-APO-1 mAb. The HTLV-1 infected (MT-2, MT-4) and *tax* expressing (C8166) cell lines used in this study showed overall increased resistance to anti-APO-1-mediated apoptosis at concentrations of mAb which produced apoptosis in Jurkat cells. The highest resistance was observed in MT4 cells which produce very low levels of virus. However, Hut78 cells which are latent in HTLV-1 expression also demonstrated less susceptibility to anti-APO-1 at the same levels which induced apoptosis in Jurkat cells. Hut78 cells represent an activated T-cell model and express the activation molecules Ia and the IL-2R (186). A reduced sensitivity to anti-APO-1-induced apoptosis in Hut78 and MT-4 cells may result from the level of activation of cellular pathways in these transformed cell lines.

As Jurkats are a resting T-cell model and also sensitive to anti-APO-1-induced apoptosis, it was important to determine whether the enhanced resistance to the effects of anti-APO-1 in HTLV-1-associated cell lines was the result of the mitogenic effect of HTLV-1 particles. Heat-inactivation of virus produced by MT-2 cells did not interfere with the ability of supernatant of these cells to transfer protection against apoptosis to Jurkat cells. While MT-4 cells are highly resistant to anti-APO-1, supernatant of these cells was not able to transfer resistance to Jurkat cells. These observations are of interest as it has recently been demonstrated that the mitogenic effect of HTLV-1 on target T-cells could not be induced by virus particles in supernatants of MT-2 or Hos/PL cells. The mitogenic effect was mediated by virus-producing cells and additionally required cell-to-cell contact (145). The ability of supernatant of the HTLV-1 *tax* expressing cell line C8166 and inactivated supernatant of MT-2 cells, which express high levels of intracellular *tax* (168), to transfer resistance suggested that this effect could be mediated by the HTLV-1 *tax* protein.

Both transfection of pRSV*tax* and culture with soluble *tax* protein conferred a degree of protection to Jurkat cells against anti-APO-1 induced apoptosis. Soluble *tax* protein does not induce proliferation of human T-cells on its own but can cooperate with other mitogenic agents to induce proliferation of T-cells (195). Mitogenicity induced by HTLV-1 expressing cell lines has not been attributed to *tax*, and it has been observed that addition of

anti-*tax* mAb to cells cultured with HTLV-1 producing cells does not inhibit the mitogenic effect (145). The enhanced resistance of *tax*-expressing cells to anti-APO-1 is not likely due to IL-2 expression as *tax* alone cannot induce efficient IL-2 expression (197, 242, 275). Furthermore, Debatin et al. (48) reported that the addition of exogenous IL-2 to ATL cell cultures did not prevent apoptosis mediated by APO-1. *Tax* is able to activate a wide range of cellular genes and may mediate its inhibitory effect on anti-APO-1-induced apoptosis by inducing the expression of genes which block the cell suicide pathway. In addition, *tax* has been identified to possess oncogenic potential *in vitro* (242, 289) and has been described as a viral oncogene acting cooperatively with the *ras* oncogene to induce neoplastic transformation of cells (242). Whether *tax* acts on its own or cooperates with other genes in effecting inhibition of apoptosis remains to be determined.

The pathway by which *tax* blocked apoptosis was investigated using a panel of reagents reported to inhibit apoptosis in other cell systems. We found that anti-APO-1-induced apoptosis in Jurkat cells could be inhibited by TPA which directly activates PKC. Zinc ions, which are inhibitory to endonuclease activity, and the chelation of Ca^{2+} in the extracellular medium by EGTA also inhibited anti-APO-1-induced apoptosis. The immunosuppressive drug cyclosporin A, and activation via the CD28 molecule, while reported to be inhibitory to apoptosis in other systems (73, 88, 92, 174), were not inhibitory to anti-APO-1-induced apoptosis. Activation of Jurkat cells via the TCR/CD3 receptor did not inhibit anti-APO-1-induced apoptosis, although Jurkat cells express high levels of CD3 and progress through the cell cycle at the levels of anti-CD3 used in our experiments. However, this mode of activation may initiate other pathways which are not induced by direct activation by TPA and ionomycin and which the anti-APO-1 pathway may require to support apoptosis. Inhibition of PKC by staurosporine was able to prime MT-2 cells to undergo apoptosis at lower concentrations of anti-APO-1. This suggests a requirement for PKC in HTLV-1 *tax*-associated inhibition of anti-APO-1-induced apoptosis.

HTLV-1 producing cells can confer IL-2-independent growth to human T-cells,

however this transforming effect may require between one week (54) to several weeks (189, 203) of co-culture. The inhibitory effect of *tax* on apoptosis may be one of several early mechanisms used by the HTLV-1-infected cell to resist elimination to support the persistence of dysregulated T-cells capable of attaining the potential to eventually develop into ATL.

Acknowledgements

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CHAPTER VII

GENERAL DISCUSSION

GENERAL DISCUSSION

Infections with human retroviruses are characterized by an asymptomatic period of variable duration prior to the clinical appearance of disease. In the case of HIV-1 infection, the T-cell population exists in a high level state of activation as is evident by the expression of related activation markers. These include β-2 microglobulin (330), serum and urinary neopterin (74), soluble IL-2R (223, 268), soluble CD8 molecules (1, 247) and soluble TNFα receptors (85, 131). The increase in many of these markers during disease progression is parallelled by the development of immune system dysfunction. The dysfunction may result in an anergic-like loss of proliferative potential (155, 156, 234, 265, 266) or may result in cell death by spontaneous or activation-induced apoptosis (8, 12, 88, 92, 204).

In contrast to the cytolytic effect of HIV-1, HTLV-1 infection may trigger the proliferation of infected cells and the development of mature T-cell cancer. During the asymptomatic period the infected cell is in a state of activation as is evident by the constitutive expression of IL-2R (49, 240, 322) and in some cases the production of IL-2 (49, 86, 101). The level of immune activation observed in HTLV-1 asymptomatic patients is not likely induced by *tax* alone as it has been previously shown that *tax* cannot activate PBLs on its own. Activation can be induced by cell-to-cell contact with HTLV-1 infected cells (145). The maintenance of this state of activation may indeed be dependent, in part, on the ability of *tax* and perhaps other viral or cellular encoded genes to upregulate cellular gene expression.

In this thesis we have investigated the *in vitro* tropism of HTLV-1 (Chapters 2 and 3). As the preferred target for infection by HTLV-1 is the T-cell, the impact of T-cell

activation pathways on HTLV-1 expression was compared with pathways mediating expression of HIV-1 and a cellular T-cell activation gene (Chapter 4). T-cell activation pathways were further explored (Chapter 5) resulting in the identification of a mechanism which may mediate HTLV latency. The abrogation of normal T-cell responses by viral mechanisms, as described in Chapter 6, combined with the aforementioned mechanism controlling viral latency have provided for a new model of disease progression in HTLV-1 infection which is presented in this discussion.

Cellular Tropism of HTLV-1

Human retrovirus infection is not restricted to T-lymphocytes. However, the resulting immune dysfunction observed in the infected individual ultimately imparts cytolytic (HIV-1) or proliferative (HTLV-1) effects upon the T_H cell population. The cellular tropism of HIV-1 and factors required to support productive infection have been reviewed in chapter 1. In the case of HTLV-1, epidemiological data have revealed that individuals infected early in life have a greater likelihood of developing ATL than TSP or HAM (211). HTLV-1 expression is high in mononuclear cells of TSP and HAM patients and expression of the virus is clearly linked to disease progression (84, 211, 324). In contrast, despite a similarity in viruses isolated from ATL, HAM and TSP patients, HTLV-1 is quiescent in leukemic cells of ATL patients (211). In addition, the development of ATL is preferred by neonatal infection via mother's milk (211). These observations emphasize the importance of identifying cell types which are permissive to HTLV-1 infection, as was described in chapter 3 of this thesis. The determination of cell types which express the putative and yet unknown receptor for HTLV-1, and which support productive infection by HTLV-1, is a necessary adjunct to studies of the regulation of virus expression. We have developed two systems to support further work on the cellular tropism of HTLV-1 and the cellular events required for the establishment of productive infection (Chapters 2 and 3). HTLV-1 can penetrate a variety of cell types (154) but few non-lymphoid cells are able to

support infection. This observation supports the view that the HTLV-1 receptor(s) may be commonly expressed on diverse cell types. An expression system in a cell type which is non-permissive to productive infection will provide a useful screening tool for the identification of putative HTLV-1 receptor molecules (Chapter 2). The cellular events which mediate productive infection by HTLV-1 have not as yet been defined. The system for detection of infectivity described in chapter 3 provides a means by which these cellular events may be identified.

The Role of T-cell Activation Pathways in Retrovirus Pathogenesis

In chapters 4 and 5 of this thesis the focus of our investigations was the T-cell and the impact of normal immune signals on retroviral expression. To accomplish this, full length LTRs of each virus linked to the reporter gene β-galactosidase were stably integrated into the genome of human Jurkat T-cells, and the response of the LTR to activation signals was measured by fluorescence detection procedures (Chapter 4). The results of this work revealed that the HIV-1 LTR was upregulated by single signals such as the direct activation of PKC or via the CD28 pathway. This type of activation was in contrast to the complete activation (PKC plus Ca^{2+}) required by the NF-AT enhancer element of the IL-2 promoter. It is important to note that activation of HIV LTR-mediated gene expression in response to antiCD3 monoclonal antibody significantly preceded that of our cellular enhancer model NF-AT. We concluded from these data that the HIV-1 LTR is likely capable of responding early during T-cell activation and, in addition, may be responsive to incomplete T-cell activation signals as generated in an immunocomprimized cell. Studies are in progress to determine whether virus expression is altered in infected cells which demonstrate anergy or apoptosis in response to activation *in vitro*.

In stark contrast to HIV-1, the HTLV-1 LTR was not induced by activation via the CD3 or CD28 pathways. In addition, the induction of the HTLV-1 LTR by phorbol ester (TPA) was inhibited by the Ca^{2+} ionophore ionomycin, suggesting that triggering of Ca^{2+}

pathways in the cell may adversely affect virus expression. Further experiments revealed that ionomycin-mediated inhibition of the HTLV-1 LTR was not restricted to TPA-induced activation mechanisms. In addition, basal (uninduced) LTR activity, *tax*-mediated activation and the synergistic induction by *tax* plus TPA were comprimized by ionomycin. In studies with Ca^{2+} chelators and inhibitors of Ca^{2+}-dependent pathways, a cyclosporin A sensitive pathway was identified to be the cause of inhibition (Chapter 5). The suppression of calcium dependent phosphatase activity by cyclosporin A recovered LTR responses in ionomycin treated cells to levels above those obtained in the absence of ionomycin. These results suggest a complementation of ionomycin and cyclosporin A in LTR induction. It may be possible that increased levels of free Ca^{2+}, generated in response to ionomycin, may provide an increased affinity of transcription factors for the LTR resulting in an enhanced LTR induction in the presence of cyclosporin A.

These results further strengthen the theory that T-cell activation might be inhibitory to HTLV-1 expression. In this respect, the presence of proviral DNA, and low levels of viral products, such as *tax*, may be sufficient for the expression of IL-2R by the infected T-cell. However, an elevated state of activation resulting in the expression of IL-2R and activation of the IL-2/IL-2R autocrine loop may concurrently provide conditions which suppress virus expression. This may provide one of likely several events which contribute to the quiescence of the virus during the asymptomatic period of ATL. A shut down in virus expression could also render the infected cell protected from elimination during routine immune surveillance, thus allowing an expansion of the population of cells expressing HTLV-1 proviral DNA. Although current evidence supports our hypothesis that T-cell activation plays a role in the different routes of pathogenesis of human retroviruses, the exact mechanisms which occur *in vivo* remain to be elucidated. The overwhelming differences found in the responses to T-cell activation pathways between HTLV-1 and HIV-1 suggest that these two viruses have evolved very different strategies for residing in the same host cell type, although they both apparently abrogate T-cell activation signals to

control their replication. These differences appear to influence the contrasting progression of HIV-1 infection to AIDS and HTLV-1 infection to ATL. However, the ability of two human retroviruses to evoke two such diverse clinical endpoints underlines the contrast in the cellular pathways which can be appropriated by these pathogens.

Viral Mechanisms Mediating Disease Progression

Although the *tax* gene has been demonstrated to have oncogenic potential (215, 242, 289, 325) and pleiotropic effects in the activation of cellular genes, less than 1% of HTLV-1-infected individuals develop ATL. In addition, virus expression is minimal during the asymptomatic stage of infection. The oncogenic potential of *tax* may thus occur only under specific cellular conditions when simultaneous *tax* expression is present. It has been proposed that HTLV-1 infection is a necessary but insufficient step and that a second rare event is required for cellular transformation. In the two-step model of progression (197, 325), continuous triggering of T-cell activation is suggested to shift the carrier to a leukemic state. However, as the progression to ATL is rare, other mechanisms have been proposed to underly the development of aggressive disease. One condition speculated to provide the machinery to support transformation during HTLV-1 infection is pre-existing host cell damage (325). The reduced expression of the DNA repair enzyme β-polymerase in human T-cell lines (125) is one example of a *tax*-mediated transcriptional event which may have detrimental effects on normal cell function. Infection of a cell with pre-existing DNA abnormalities, combined with an impaired DNA-repair mechanism, might provide two cooperative events for the progression to leukemogenesis. This model is strengthened by the observation that a variety of different chromosomal abnormalities are frequently found in ATL patients (28, 77, 207). The infected cell may also be at a higher risk of *tax*-induced transformation due to the maintenance of oxidative stress within the cell by a persistent expression of ATL derived factor (ADF), the human homologue of the bacterial coenzyme thioredoxin (110, 321). Oxidative stress has also been proposed as one of several cellular

conditions significant in the progression to AIDS (250, 259).

Immune dysfunction is a characteristic common to human retrovirus infection. One mechanism suggested to account for the loss of T_H-cells during progression to AIDS, other than direct virus-mediated destruction, centers on the dysfunctional activation of the HIV-infected T-cell. Prior to a significant decline in CD4+ cells, immune dysfunction is evident (155, 156, 158, 235, 271, 277, 315), demonstrated by a suppression of activation in response to signalling by antigen, mitogen and pokeweed mitogen (155, 156, 234, 265, 266). One outcome of *in vitro* activation of the CD4 and CD8 cells of HIV-1 infected individuals is apoptosis (8, 12, 88, 92, 204). The cell becomes programmed for death characterized by activation of a Ca^{2+}-dependent endogenous endonuclease which cleaves chromosomal DNA between nucleosomes (267, 319). In chapter 6, the induction of apoptosis in HTLV-1 infected cells was studied. In this work the monoclonal antibody anti-APO-1 was used to induce a high level of apoptosis in Jurkat T-cells. Cell lines infected with HTLV-1 or expressing *tax* were protected from apoptosis. This protection was conferred by the HTLV-1 *tax* protein and showed a dependence on the basal PKC production in the infected cells. Virus-mediated inhibition of apoptosis has been observed for adeno (245), Epstein Barr (104) and Sindbis (165) viruses. The dysregulation of cell suicide pathways by virus infection favours the establishment of viral integration and subsequent expression. Thus HTLV-1 infected cells may evade normal host mechanisms utilized to remove unwanted or inappropriately responsive cells by the host.

Model of HTLV-1 Pathogenesis

The most frequently proposed theory for progression to ATL is based on a two-step activation model (197, 325). During step 1 of this model, the infected cell becomes predisposed to autonomous growth. This condition would be supported by weak functioning of either the autocrine or paracrine IL-2/IL-2R loop. This scenario is supported by the strong induction of IL-2R by *tax* (46, 164, 227), while efficient activation of IL-2

by *tax* has been reported to require additional stimuli (197, 242, 275). During step 1, virus expressing cells would be removed by immune surveillance while antigen-negative cells could again enter a second cycle of proliferation. These cycles would need to be repeated for 20 to 30 years in a healthy carrier to support the asymptomatic period.

The two-step activation model does not explain how virus expression is minimized during the asymptomatic period. In step two of this model, antigen presentation is suggested as the necessary signal for progression from a carrier to a leukemic state. However, this rationale is disputable given the evidence described in this thesis of the inhibition of virus expression by Ca^{2+}-dependent T-cell activation. In light of the work presented in this thesis, a new model of HTLV-1 pathogensis may be proposed. Following infection of T-cells by HTLV-1, the IL-2/IL-2R autocrine loop becomes established as described by step one of the two-step activation model. Virus activity, despite IL-2R expression, is minimal and may result from a block in virus expression by Ca^{2+}-dependent phosphatase activity, induced by the same pathway required for IL-2R expression. While low levels of virus expression may continue in some cells to support the IL-2/IL-2R autocrine loop during the asymptomatic phase of infection, these cells may escape removal by programmed cell death mechanisms due to a protection afforded by the virus. The protection may be mediated directly by *tax*, may be the result of the activation of PKC within the cell or perhaps may be supported by the indirect effect of *tax* on cellular gene expression (ie IL-2R, ADF). This model imparts new information on how the quiescence of the virus may be mediated by T-cell activation and further provides a means by which the asymptomatic phase of infection may be maintained. The decreased expression of CD3 on leukemic cells (114, 298, 327) may suggest that T-cell activation occurs more continuously in infected cells, perhaps during the asymptomatic period. Since infected cells respond indiscriminately to antigen, and with no HLA-DR restriction (239), these events applied to our model would support viral latency. Protection from apoptosis may be one mechanism favouring the expansion of the infected population. The event or events that

trigger the onset of the progression to ATL remain unknown. Because progression to ATL occurs infrequently in infected individuals, the events that trigger progression may be rare. In addition, it seems likely that, as with TSP and HAM, higher virus expression may be required for progression to disease. It must also be considered that certain events may need to occur in a specific contingency of sequence during infection. The observation that T-cell activation limits virus expression, may indicate that a change in the activation potential of the cell during the asymptomatic phase could be a rare event required for the progression to ATL. A focus on understanding T-cell function during infection is essential to further our knowledge of human retrovirus-mediated disease.

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SUMMARY/SAMENVATTING

SUMMARY

This thesis describes the effect of T-cell activation signals on HTLV-1 and HIV-1 expression. Comparative studies revealed that the HTLV-1 LTR was responsive to a much narrower range of activation pathways than the HIV-1 LTR. Further investigations were aimed at identifying the activation pathways inhibitory to HTLV-1 and understanding how the presence of the virus might affect normal responses to activation.

Chapter 1 summarizes the current literature describing the transcriptional requirements of human retroviruses, with a particular focus upon T-cell activation-mediated transcription.

In chapter 2, a rapid integrated assay was developed in HeLa cells to measure HTLV-1 LTR-mediated expression. In this assay expression was measured by β-galactosidase expression directed by the LTR. This work provided a more rapid and relevant assay of HTLV-1 gene expression than transient transfection and revealed an inhibitory effect of calcium ionophore on LTR activation.

The *in vitro* cellular tropism of HTLV-1 was investigated in chapter 3. Using a recombinant virus expressing a drug resistance (neomycin) gene, infection by HTLV-1 was detectable by the transfer of neomycin resistance to target cells. The results of this investigation revealed that HTLV-1 infection can be supported by two fibroblast cell lines (Cos7, RD4) previously reported to be resistant to infection. Productive infection can be detected much more quickly using this system than with other reported infectivity assays. Because this assay provides a measure of productive infection, it will be a valuable tool for investigating cellular events which are required post-binding to facilitate productive infection.

Chapter 4 compares the responses of integrated HIV-1 and HTLV-1 LTRs to T-cell

activation signals. A cellular enhancer of the IL-2 promoter which binds the activation dependent protein NF-AT was included to permit a further comparison between signals used by retroviruses and T-cell genes. The HIV-1 LTR was found to respond to TCR/CD3-mediated activation similarly to the NFAT enhancer. However, the HIV-1 LTR did not show a dependence upon full T-cell activation, but instead was able to utilize single signals including the CD28 pathway. Thus it is possible that the HIV-1 LTR is responsive in an aberrantly activated or functionally comprimised T-cell. The LTR of HTLV-1, was not responsive to triggering of TCR/CD3 or CD28 pathways. In addition, activation induced by phorbol ester was markedly reduced in the presence of calcium ionophore. While the HIV-1 LTR has adapted to utilize activation signals commonly employed by cellular gene promoters, HTLV-1 appears to depend upon other, as yet unidentified, cellular events in its activation.

The inhibitory function of calcium ionophore on the activation of the HTLV-1 LTR was explored in chapter 5. The ionophore ionomycin reduced the level of basal (uninduced) LTR activity in T-cells and also reduced the level of *trans*-activation induced by phorbol ester, the HTLV-1 *tax* protein and full virus production. Using calcium chelators and an inhibitor of calcium-dependent T-cell activation (cyclosporin A), it was demonstrated that the actual levels of free calcium generated by ionomycin did not significantly affect LTR activation. However, the immunosuppressive drug cyclosporin A was able to overcome ionomycin-induced inhibition. Thus an early T-cell activation event requiring Ca^{2+}-dependent phosphatases, which are specifically blocked by cyclosporin A, can inhibit HTLV-1 expression. This observation may provide insight into the long asymptomatic period characteristic of HTLV-1 infection.

Activation-induced apoptosis of HIV-1-infected T-cells has been proposed as one mechanism underlying T cell loss as infection progresses. In Chapter 6, activation-induced apoptosis was explored in T-cell lines infected with HTLV-1. These experiments were designed to examine the cellular pathways controlling proliferation and underlying the

protective role of HTLV-1 infection against apoptosis suicide pathways as a possible early mechanism of HTLV-1 leukemogenesis. As a model to study this phenomenon, apoptosis was induced by the monoclonal antibody anti-APO-1 which induced a high level of apoptosis in T cells capable of undergoing activation *in vitro*. While low levels of anti-APO-1 induced widespread apoptosis in uninfected cells, HTLV-1-infected cells were found to be more resistant. Furthermore our studies revealed that protection from apoptosis was mediated by the HTLV-1 *tax* protein and was protein kinase C dependent. The results of this study suggest that *tax* protects against normal host mechanisms for removal of unwanted T cells. This phenomenon may play a role in allowing an expansion of dysregulated T cells as an early step in the development of mature T-cell cancer.

SAMENVATTING

De effecten van T-cel activatie signalen op HTLV-I en HIV-1 expressie, worden in dit proefschrift bestudeerd met behulp van een nieuwe expressie assay. Deze expressie assay is gebaseerd op een geintegreerde LTR gekoppeld aan een reporter gen. Vergelijkende studies hebben uitgewezen dat de HTLV-1 LTR op minder signaal transductie mechanismen reageert dan de HIV-1 LTR. Andere studies waren gericht op het identificeren van activatie mechanismen die HTLV-1 remmen en inzicht geven in hoe aanwezigheid van virus de normale cellulaire respons op activatie beinvloed.

Hoofdstuk 1 is een samenvatting van de recente literatuur over de voorwaarden voor transcriptie van humane retrovirussen, waarbij de nadruk ligt op de T-cel activatie gemedieerde transcriptie. Tevens worden virus-geinduceerde veranderingen in de cellulaire fysiologie tijdens ziekte progressie besproken.

In hoofdstuk 2 werd een geintegreerde assay in HeLa cellen ontwikkeld om HTLV-1 gemedieerde LTR-expressie te kunnen meten. Deze assay kwantificeert beta-galactosidase expressie onder controle van de LTR. Deze methode bleek een snellere en meer relevante assay voor HTLV-I gen expressie te zijn dan transient transfectie. Een remmend effect van calcium ionofoor (ionomycine) op LTR activatie werd in deze assay gevonden.

Het *in vitro* cellulaire tropisme van HTLV-I werd onderzocht in hoofdstuk 3. Door gebruik te maken van een recombinant virus dat een resistentie-gen voor een antibioticum (neomycine) tot expressie brengt, kon HTLV-I infectie gedetecteerd worden door het verkrijgen van neomycine resistente doelwit cellen. De studie toont een tropisme voor de fibroblasten lijnen, RD4 en Cos7, die voorheen als resistent werden beschouwd. Met dit systeem werd sneller een productieve infectie gedetecteerd dan met andere beschreven

infectie assays. Omdat deze assay produktieve infectie aantoont, zal het een nuttig hulpmiddel zijn bij het onderzoeken van cellulaire processen die nodig zijn na binding van het virus (aan de receptor) om produktieve infectie te vergemakkelijken.

In Hoofdstuk 4 worden de responsen van de geintegreerde HIV-1 LTR en HTLV-I LTR op T-cel activatie signalen vergeleken. Een cellulaire enhancer van de IL-2 promotor dat het activatie-afhankelijke eiwit NF-AT bindt werd tevens bestudeerd om een verdere vergelijking te kunnen maken tussen de signalen gebruikt door retrovirussen en T-cel genen. De HIV-1 LTR bleek op een vergelijkbare manier te reageren als de NF-AT enhancer op TCR/CD3 stimulering. De HIV-1 LTR bleek niet afhankelijk te zijn van volledige T-cel activatie, maar was daarentegen in staat om enkelvoudige signalen te benutten, inclusief de route van CD28 stimulering. De HIV-1 LTR zou dus nog responderen in een niet-adequaat geactiveerde of functioneel gecompromitteerde T-cel. De LTR van HTLV-I werd niet geactiveerd via triggering van het T-cel receptor/CD3 complex of het CD28 molecuul. Bovendien werd phorbol-ester geinduceerde activatie sterk gereduceerd in de aanwezigheid van calcium ionofoor (ionomycine). Terwijl de HIV-1 LTR zich heeft aangepast om de fysiologische activatie signalen van cellulaire genpromotors te benutten, lijkt de LTR van HTLV-I voor activatie afhankelijk te zijn van andere nog niet geidentificeerde cellulaire processen. Tevens werd in deze studies aangetoond dat de toepassing van een geintegreerde LTR een gevoeliger en nauwkeuriger meting van triggering van celmembraan determinanten mogelijk maakt dan de transiente transfectie met LTR-reporter gen vectoren.

De remmende werking van calcium ionofoor (ionomycine) op de activatie van de HTLV-I LTR werd onderzocht in hoofdstuk 5. De ionofoor ionomycine reduceerde het basale niveau (niet-geinduceerd) van LTR-activiteit in T-cellen en tevens het niveau van *trans*-activatie geinduceerd door phorbol-ester, het HTLV-I *tax* eiwit en volledige virus productie. Met behulp van calcium chelatoren en een remmer van de calcium-afhankelijke T-cel activatie (cyclosporine A) werd aangetoond dat de niveaus van vrij calcium gegenereerd

door ionomycin geen significante invloed hadden op de activatie van de LTR. Echter, de immunosuppresivum cyclosporine A was in staat om de ionomycin-geinduceerde remming van de phorbol ester en *tax* LTR activiteit teniet te doen. Blijkbaar kan HTLV-I expressie geremd worden door een vroege gebeurtenis in T-cel activatie, waarbij Ca2+-afhankelijke fosfatases betrokken zijn die specifiek geblokkeerd worden door cyclosporine A. Deze observatie kan mogelijk inzicht verschaffen in de mechanismen die ten grondslag liggen aan de lange asymptomatische periode na HTLV-I infectie.

Activatie-geinduceerde apoptosis van HIV-1 geinfecteerde cellen is mogelijk een van de mechanismen verantwoordelijk voor de afname van T cellen gedurende infectie. In hoofdstuk 6 werd activatie-geinduceerde apoptosis in HTLV-I geinfecteerde T-cellijnen onderzocht. Deze experimenten werden opgezet om de cellulaire mechanismen op helderen die proliferatie controleren en welke ten grondslag liggen aan de beschermende rol van HTLV-1 tegen het apoptosis 'zelfmoord' programma, hetgeen een mogelijk vroeg mechanisme in HTLV-1 leukemogenesis kan zijn. Apoptose werd geinduceerd door het monoclonale antilichaam anti-APO-1 dat een hoog percentage apoptose veroorzaakt in T cellen die het vermogen hebben *in vitro* geactiveerd te worden. Terwijl lage concentraties van anti-APO-1 efficient apoptose induceerden in niet-geinfecteerde cellen, waren HTLV-I geinfecteerde cellen en cellen die HTLV-I *tax* tot expressie brengen resistent tegen apoptose. De resultaten van dit onderzoek tonen aan dat de *tax*-gemedieerde remming van apoptose protein kinase C afhankelijk is. De resultaten van deze studie doen vermoeden dat *tax* beschermt tegen normale mechanismen voor het verwijderen van niet gewenste cellen. Inhibitie van apoptose door *tax* is een mogelijk mechanisme ter ondersteuning van de expansie van HTLV-1 ontregelde T-cellen als een vroege stap in de ontwikkeling van T-cel kanker.

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