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ROLE OF THE CD4 RECEPTOR IN HUMAN T LYMPHOCYTE SIGNALLING: EFFECTS OF HIV-1 INFECTION ON HUMAN T CELL ACTIVATION* (U)

C. H. June, LCDR, MC, USN, M.D.[+], G. P. Linette[#], M. C. Fletcher, Ph.D.[+], and R. J. Hartzman, CAPT, MC, USN, M.D.[+]

[+]Naval Medical Research Institute
Bethesda, Maryland 20814
[#]Georgetown University School of Medicine
Washington, DC 20007

ABSTRACT

It has previously been shown that infection of human CD4 cells with human immunodeficiency virus (HIV-1) results in the establishment of a chronic, non-cytopathic infection. We have recently found that cells infected with HIV-1 exhibit a specific impairment of cellular signalling. After polyclonal stimulation of the CD3/T cell receptor complex by anti-CD3 monoclonal antibody, the cells have a blunted or absent increase in the intracellular free calcium concentration ($[Ca^{2+}]i$) within the first three minutes after stimulation. This impairment after antigen specific stimulation is not the result of decreased viability or cellular wellness because the response to stimulation of the CD2 (E rosette receptor or LFA-3 ligand) antigen remains intact. In the present study we have investigated the mechanism of the effect on cellular signalling by HIV-1. CD4 T cells were infected with HIV-1 and labeled with myoinositol. Inositol phosphate production was impaired after CD3 stimulation, suggesting that the effect of HIV-1 occurs at or before the activation of phosphoinositide specific phospholipase C. Recent studies have shown that the CD4 molecule is involved in cellular signalling with the T cell receptor as well as functioning as a receptor for MHC class II antigen and as the receptor for the HIV-1 virus. To further investigate the effects of HIV-1 on cellular signalling, the CEM leukemic T cell line was chronically infected with HIV-1, or a spontaneous mutant strain of HIV-1 that fails to cause modulation of the CD4 antigen. Calcium mobilization was impaired in cells infected with both strains of virus, indicating that impaired signalling is not simply the result of decreased surface expression of the CD4 receptor.

INTRODUCTION

The elucidation of the immediate molecular events that accompany lymphocyte activation is an important area of investigation because these events are logical control points for cellular activation and thus represent potential immunopathogenetic mechanisms and are prime targets to modulate the immune response therapeutically. There are multiple biochemical cascades that are initiated during T cell activation; these include (but are not limited to) increases in cytosolic calcium concentration, activation of protein kinases, alterations of cytosolic cyclic nucleotide concentrations, and alterations of membrane permeability to sodium, protons, and potassium. According to the current paradigm, the autocrine production of IL-2 by T cells requires the combined effects of an increased intracellular ionized calcium and protein kinase C activation (1). T cells may be activated to proliferate and produce IL-2 after "membrane bypass" activation provided by treatment of cells with calcium ionophore to elevate cytosolic free calcium concentration and phorbol esters to activate protein kinase C (2-4). Ligation of the T cell antigen receptor and the associated CD3 complex, either by antigen or by monoclonal antibodies (mAb), has been shown to cause both activation of protein kinase C and elevation of intracellular calcium (5-7). It is not yet known what are the minimal and essential signals required to cause a resting T cell to enter the cell cycle. For example, after anti-CD3 stimulation the following events have been shown to occur within 1 minute of stimulation: hydrolysis of phosphatidyl inositol bis-phosphate with production of Ins(1,4,5)IP3 and diacylglycerol; increased cytosolic calcium concentration from cellular stores and transmembrane transport; increased cAMP concentration; cytosol to membrane translocation of protein kinase C; and activation of protein tyrosine kinase(s) (7-12).

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The elimination or impairment of the CD4 T cell would be expected to have global effects on the mature immune system. The CD4 cell has many effector functions that include, but are not limited to, antigen specific recognition with subsequent clonal expansion and lymphokine/cytokine production. These effector functions have been previously described in immunologic terms as a variety of diverse processes including delayed type hypersensitivity, allograft rejection, helper function for B cell immunoglobulin production, and helper function for the induction of killer T cells. The CD4 cell is a major source of lymphokines (IL-2, IL-3, IL-4, IL-5, GM-CSF, gamma-interferon, tumor necrosis factor alpha, and tumor necrosis factor beta).

The constellation of symptoms and signs resulting from infection with the human immunodeficiency virus (HIV) has been termed the acquired immunodeficiency syndrome (AIDS). Infection by this virus is nearly always fatal, although there is often a prolonged latent period of 7 to 8 years before symptoms appear (13-15). The most conspicuous abnormality associated with HIV-1 infection is a quantitative deficiency of the CD4 helper/inducer T cell (16,17). The fact that the CD4 T lymphocyte has a central role in the immune and hematopoietic systems accounts, in part, for the wide range of immunologic defects reported with HIV infection.

There is increasing evidence that functional impairments of CD4 cells, in addition to depletion of CD4 cells occurs in patients with AIDS. Cutaneous anergy, i.e. lack of response to recall antigens, is one of the earliest immunologic abnormalities to occur in HIV-infected individuals (18). It was found that a striking impairment of proliferation of peripheral blood lymphocytes from HIV-1 infected patients occurred after stimulation with antigen (19). However, from those studies it was not possible to determine if proliferation was impaired due to a loss of cells with proliferative capacity or if proliferation was impaired only after cellular activation by a distinct stimulus. Evidence for a selective defect in soluble antigen recognition was shown by Lane et al where purified CD4 cells from patients with AIDS had decreased proliferation on a per cell basis after stimulation with tetanus toxoid, while proliferation of the cells after polyclonal stimulation by the lectin pokeweed mitogen was intact (20). Several other studies have found defects in antigen-driven lymphocyte proliferation in HIV-infected patients (21-23). Impaired T cell proliferation to recall antigens is a predictive factor for patients who are more likely to develop opportunistic infections (24). This defect may be important in the pathogenesis of AIDS because it occurs in patients infected with HIV-1 who are asymptomatic. It is intriguing to speculate that the basis of this defect is related to the signaling defect that we have observed in vitro in the cells that survive the acute cytopathic effects of HIV (25).

The T cell antigen receptor consists of a heterodimeric complex composed of either alpha/beta chains or gamma/delta chains (2). The receptor is non-covalently coupled to the CD3 complex, a non-polymorphic structure that consists of polypeptides that are encoded by 5 genes: CD3 gamma, delta, epsilon, zeta and eta. Antigen recognition occurs through the T cell receptor and it is thought that signal transduction occurs through the CD3 complex (3,5). CD2 is a 50 kD molecule encoded by chromosome 1 that is expressed on all human peripheral blood T cells and most natural killer cells but not on B lymphocytes. The CD2 molecule was first defined as the E rosette receptor and more recently found to be a ligand for the LFA-3 molecule (26). We found that the cells that survive infection by HIV-1 have impaired calcium signalling after CD3 stimulation and not after CD2 stimulation (25).

The CD4 molecule has been shown to facilitate interactions with other cells bearing HLA class II antigens, to function as a signal transducing molecule, and to serve as a receptor for HIV (reviewed in [27]). It was originally thought that CD4 facilitated cellular activation by functioning as a cellular adhesion molecule or that it could transmit signals that down-regulated activation through the CD3/TCR complex (28). Recently, however, we and others found that the CD4 molecule transmits a potent activating signal when it is cross-linked in association with CD3 (29,30). In addition, it was recently published that gp120 itself could directly activate T cells to cause calcium mobilization and phosphatidyl inositol hydrolysis (31). However, we do not find evidence to support the claim for direct activation of CD4 cells by gp120 (32), and believe that the results of Kornfeld et al are most likely explained by aggregates of gp120 that cross-link CD4, and therefore, cause calcium mobilization (12). Phorbol esters and antigen stimulation induce rapid phosphorylation and internalization of CD4, however these events are not required for HIV infection because treatment of T cells with gp120

does not cause phosphorylation of CD4 (33) and because cells expressing mutants in the cytoplasmic domain of CD4 that prevent internalization of CD4 remain susceptible to infection with HIV-1

MATERIALS AND METHODS

VIRUS AND CELL LINES. The CEM-T4 human T cell leukemia line was obtained from the AIDS Research and Reference Reagent Program of the National Institutes of Health; this line is highly permissive for HIV-1 infection. The CEM-T4 line is CD2, CD3, and CD4 positive but has a defect in the cell surface expression of the CD3/TCR complex. If the cells were treated with Phorbol Mysteric Acid (PMA), then all cells had easily detectable CD3. Only ½25% of our wild type CEM cells had calcium mobilization after CD3 stimulation. To circumvent this difficulty, we stimulated indo-1 loaded CEM cells with anti-CD3 and electronically sorted on the basis of calcium response (34). After three successive rounds we isolated CEM clone A1 that was homogenous with respect to CD3 surface expression and where ¶ 85% respond with calcium mobilization after CD3 stimulation. This phenotype was maintained for ½2 months of continuous culture. Furthermore, these cells develop a chronic infection after HIV-1-infection, and chronically infected cells have impaired calcium signaling after CD3 stimulation.

For experiments using primary CD4 cells, buffy coats were obtained by phlebotomy or leukopheresis of healthy donors. Peripheral blood lymphocytes were isolated from the buffy coat by Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) density gradient centrifugation. Monocytes were depleted from the PBL by density gradient centrifugation (Sepracell). The $CD4^+$ subset of T cells was then isolated from the monocyte-depleted cells by negative selection using immunoabsorption as previously described (25,35).

To infect primary CD4 cells, cells were activated by a 3-day culture in tissue flasks coated with CD3 mAb, and incubated with cell-free virus at a multiplicity of infection of 0.1 as previously described (25). To obtain chronically infected cell lines, CEM clone A1 was cultured with irradiated SupT1 (a leukemic T cell line, gift from J. A. Hoxie, University of Penn.) cells that were infected with HIV-1 IIIB (LAV, gift from T. Folks, NIAID, NIH) or A1.9.5-5 (gift from J. A. Hoxie) virus. In both primary cell cultures, and the cell lines infected with IIIB, syncytia and CPE (cytopathic effect) appears on ½day 7 and RT (reverse transcriptase) peaks at day 7 to 9; at this time live cells were separated from dead cells by density gradient centrifugation and the surviving cells cultured. In cells infected with A1.9.5-5, there was no syncytia formation, and little or no cellular death occurred. Virus production was monitored by measuring reverse transcriptase activity in culture supernatants, by indirect immunofluorescence analysis of viral antigens with HIV immune globulin in cells fixed in acetone-methanol and by an antigen capture assay for p24 that is sensitive to ½1 pg/well (36).

ANALYSIS OF INTRACELLULAR IONIZED CALCIUM CONCENTRATION BY FLOW CYTOMETRY. Our procedure for the measurement of $[Ca^{2+}]i$ in single cells has been described in detail elsewhere (34,37). The technique permits the simultaneous measurement of $[Ca^{2+}]i$ and cell surface antigens in large numbers of single cells, and is capable of detecting a calcium response in as few as 0.3% of cells analyzed.

PHOSPHOINOSITOL METABOLISM. We have recen[illegible]apted the method originally described by Berridge et al. (38) for the separation of i[illegible]itol polyphosphates by anion exchange chromatography using an automated FPLC system (Pharmacia) with an HR5/5 mono Q anion exchange column linked to a continuous flow in-line liquid scintillation counter (Radiomatic model IC) for quantitation of the inositol phosphates. This system offers the advantages of i) improved resolution of the inositol phosphates (for example, 1,3,4,5-IP_4 is readily identified), and ii) more rapid analysis due to the presence of the inline radioactive flow monitor.

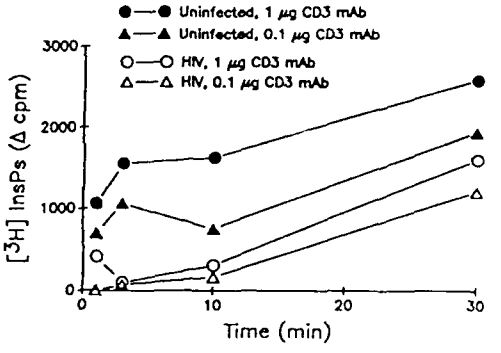
The assay of inositol phosphate formation used purified CD4 T cells or T leukemia cell lines that were cultured in the presence of 10 uCi/mL [^{3}H]- myoinositol (SA 20 Ci/mmol, NEN). We have not used inositol-depleted medium due to concerns that inositol pools may be disturbed. The cells were washed extensively in Hanks Balanced Salt Solution (HBSS) without phenol red, immediately prior to assay to remove unincorporated [^{3}H]- myoinositol and residual phenol red.

The cells (5 x 10^6)/sample were equilibrated at 37°C in lithium-free medium, stimulated with CD2 or CD3, pelleted, lysed and water soluble inositol phosphates separated from the polyphosphoinositides by chloroform:methanol as described (38). The samples were dried under N_2, and the samples frozen until analysis by FPLC. The binary buffer system in which the inositol phosphates were separated consisted of Buffer A, 10 mM HEPES, 0.1 mM EDTA, 0.1 mM $ZnSO_4$, pH 7.4, and Buffer B which was Buffer A supplemented with 0.5 M Na_2SO_4 (39).

RESULTS

STUDIES ON THE EFFECTS OF CD2 AND CD3 STIMULATION ON PHOSPHOINOSITOL METABOLISM. We have performed studies using standard anion-exchange column chromatography of the effects of HIV infection on polyphosphoinositol metabolism (Fig 1.). These studies show a clear reduction in the total production of inositol phosphates after CD3 stimulation of HIV-1-infected cells compared to uninfected cells. This technique is unable to distinguish Ins(1,4,5)P3 from Ins(1,3,4)P3, and IP3 from IP4; for this reason, we have decided to use high resolution FPLC chromatography to further study the effects of HIV on cellular signalling. In addition, we have done detailed studies on normal, uninfected T cells to characterize the biochemical signals induced by CD2 and CD3 signalling. The results shown in fig 1 were from cells treated with lithium, used in order to prevent inositol recycling, and therefore, results in accumulation of IP1. In all subsequent experiments, although technically more demanding, we have abandoned the use of lithium because of potentially adverse effects it may have on signalling (40).

Fig 1. Effects of HIV-infection on total water soluble inositol phosphate production after CD3 stimulation. CD4 blasts were isolated, infected with HIV- 1 and cultured as described (25). On day 18 of culture, cells surviving HIV infection and control uninfected CD4 cells were labeled with 20 uCi/ml ^{3}[H]- myoinositol (specific activity 10-20 Ci/mmol, NEN) for 12 hr in inositol-free EMEM. The cells were washed extensively, resuspended (1 x 10^7 cells/ml) in RPMI containing 5% FCS and 10 mM LiCl and incubated at 37 deg for 30 min. Cells (2 x 10^6) were stimulated with CD3 mAb 38.1 at optimal and suboptimal concentrations or a control mAb. At various times, the reaction was stopped by addition of chloroform and methanol. The aqueous phase was recovered, applied to a Dowex column and total inositol phosphates eluted using 1 M Formate/0.1 N formic acid (38). The cpm eluted from unstimulated cells (500 to 800) was subtracted at each time point; the average of duplicate samples at each point is shown.



Studies by other laboratories using uninfected leukemic cell lines have not demonstrated any biochemical differences between CD2 and CD3 stimulation. However, in view of our recent finding of the difference in calcium signalling between uninfected cells and cells infected with HIV-1 (25), and in view of recent findings concerning tyrosine phosphorylation of the CD3 receptor zeta chain after CD2 stimulation but not CD3 stimulation (41), we are exploring a potential biochemical basis for differences between the patterns of calcium response of T cells after CD2 and CD3 stimulation. Because one of our goals is to study the effects of CD2 and CD3 stimulation on inositol metabolism in HIV-1-infected cell lines and in primary cultures of CD4 cells, we are carefully examining the effects that stimulation with different combinations of CD2 and CD3 mAbs might have on PI turnover. In Table I, the response of InsP, Ins(1,4)P2, Ins(1,4,5)P3, and Ins(1,3,4,5)P4 after stimulation of the cells with CD3 mAb 38.1 (IgM) and CD3 mAb G19-4 (IgG1) is shown . This can be compared to the time course of [Ca2+]i changes determined at the same time with a portion of the myoinositol- loaded cells that were also labeled with indo-1 (fig. 2). Antibody 38.1 (used in the study that showed a signalling defect in the CD4 cells that survive HIV-1 infection) caused a rapid but transient calcium mobilization while mAb G19-4 caused a slower calcium peak. The peak InsP3 levels correlate well with the time of onset of calcium elevation.

Table I

Effects of CD3 mAbs 38.1 and G19-4 on InsP production

Stimulus	Time (in)	InsP Production (DPM) InsP	InsP2	InsP3	InsP4
CD3 38.1	0.25	679	705	0	160
CD3 G19-4	0.25	812	0	0	5
CD3 38.1	0.75	1076	1447	1589	2068
CD3 G19-4	0.75	666	1195	1376	1150
CD3 38.1	1.5	4059	4043	3992	4210
CD3 G19-4	1.5	5547	2528	3101	3990
CD3 38.1	3.0	7018	3096	1593	3155
CD3 G19-4	3.0	9442	3448	1902	3559
CD3 38.1	10.0	6652	2846	1181	3051
CD3 G19-4	10.0	10946	3498	1058	3031
CD3 38.1	30.0	9706	897	980	1592
CD3 G19-4	30.0	4726	1655	1112	2385

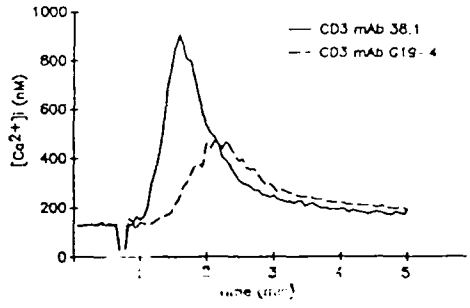


Table I and Fig. 2. Effects of CD3 stimulation by mAbs 38.1 and G19-4 on InsP production and [Ca2+]i. The CD28-positive subset of T cells was isolated by negative selection using magnetic immunobead sorting as described (25,35). T cell blasts were generated by culture on plastic-immobilized CD3 mAb for 3 days, followed by culture in medium containing IL-2 and [3]- H myoinositol (10 uCi/ml) for 5 additional days. The cells were washed and stimulated with CD3 mAb 38.1 1 ul ascites or CD3 mAb G19-4 10ug/ml. These doses of antibodies were shown in previous experiments to cause maximal calcium mobilization (25). Inositol phosphates were separated by FPLC, and the dpm displayed for the integrated peaks of InsP, Ins(1,4)IP2, Ins(1,4,5)IP3, and Ins(1,3,4,5)IP4. Simultaneous with the inositol phosphate analysis, a portion of the inositol labeled cells was loaded with indo-1 and changes in [Ca2+]i determined by flow cytometry after CD3 stimulation experiment (Fig. 2); mabs were added at the gap in analysis at t=0.75 min. Cells were analyzed at 150 cells/sec and the mean [Ca2+]i shown (25).

A number of laboratories have reported that various pairwise combinations of CD2 mAbs are mitogenic and cause calcium mobilization (6, 41-45). We found that the CD2 calcium signal had a characteristic delayed pattern (6). In order to study the mechanism for the preservation of the CD2 signal in HIV-infected cells, it was first necessary to establish if there were differences in uninfected cells between the CD2 calcium signals elicited by different modes of CD2 stimulation. In Table II and Fig.3 we have compared the effects of stimulation of T cells with the mitogenic combination of CD2 mAb 9.6 + 9-1 versus biotinylated 9.6 + avidin (9.6 BA). We have previously shown that binding of the CD2 9-1 epitope is not required for calcium mobilization, and that crosslinking of the CD2 9.6/$T11_1$ epitope is sufficient for calcium mobilization (42). It can be seen that the calcium signal was more rapid after CD2 9.6 BA than after 9.6 + 9-1 stimulation (Fig 3), although the magnitude of the signal was similar. In contrast, there was a marked difference in InsP production; the 9.6 + 9-1 signal caused much more prolonged elevation of inositol phosphates (Table II). It is possible that this difference in PI signalling explains why the 9.6 + 9-1 system is mitogenic while cells stimulated with mAb to the CD2 $T11_1$ epitope and crosslinked with anti-immunoglobulin have minimal proliferation (12,42).

Table II

Effects of CD2 mAbs 9.6 plus 9-1 and 9.6 biotin-avidin on InsP production

Stimulus	Time (min)	InsP Production (DPM) InsP	InsP2	InsP3	InsP4
CD2 9.6 BA	0.25	118	703	1218	0
CD2 9.6 +9-1	0.25	0	0	179	0
CD2 9.6 BA	0.75	558	1334	2625	688
CD2 9.6 +9-1	0.75	131	1057	12	357
CD2 9.6 BA	1.5	2193	1912	2576	1840
CD2 9.6 +9-1	1.5	307	432	501	562
CD2 9.6 BA	3.0	6071	3127	2173	2154
CD2 9.6 +9-1	3.0	3760	2038	2237	1865
CD2 9.6 BA	5.0	7492	1984	1022	1326
CD2 9.6 +9-1	5.0	9771	3693	2747	3427
CD2 9.6 BA	10.0	3710	1190	756	1049
CD2 9.6 +9-1	10.0	16902	5562	3463	4090
CD2 9.6 BA	15.0	0	167	0	308
CD2 9.6 +9-1	15.0	25804	3989	2814	2542

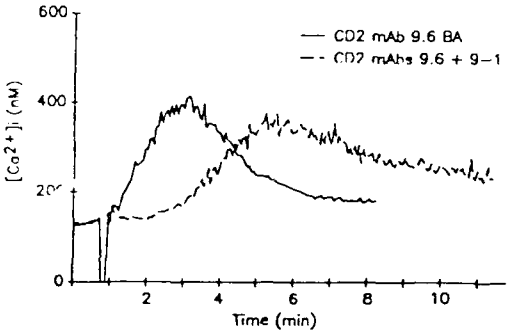


Table II and Fig. 3. Effects of CD2 stimulation by mAbs 9.6 plus 9-1 and biotinylated mAb 9.6 crosslinked by avidin on InsP production and [Ca2+]i. Cells were isolated and loaded with [3]-H myoinositol as described for Table I. The cells were washed and stimulated with CD2 mAb 9.6 10 ug/ml (time=-5 min) followed by mAb 9-1 (10 ug/ml) (time=0) or by biotinylated CD2 antibody 9.6 10 ug/ml (time= -5) followed by avidin 25 ug/ml (time= 0) in lithium- free medium. These doses of antibodies were shown in previous experiments to cause maximal calcium mobilization (25). Inositol phosphates were separated by FPLC, and the dpm displayed for the integrated peaks of InsP, Ins(1,4)IP2, Ins(1,4,5)IP3, and Ins(1,3,4,5)IP4. The DPM in control samples done at 0.25, 5 and 15 min were subtracted from the antibody treated samples. Simultaneous with the inositol phosphate analysis, a portion of the inositol labeled cells was loaded with indo-1 and changes in [Ca2+]i determined by flow cytometry after CD2 stimulation as per the InsP experiment (Fig. 3).

We next directly compared the effects of CD2 and CD3 stimulation on inositol lipid metabolism. T cell blasts were loaded with 3[H]-myoinositol and stimulated (in lithium-free medium) with CD3 mAb 38.1 or with biotinylated CD2 mAb 9.6 followed by avidin (Fig. 4). Water soluble inositol phosphates were extracted and analyzed by FPLC. Calcium mobilization studies from an aliquot of these cells that were subsequently loaded with indo-1 and stimulated with anti-CD2 and anti-CD3 show the typical rapid onset and decay of the signal after CD3 stimulation and the slow and prolonged calcium signal after CD2 stimulation (Fig. 4). These results show that a rapid burst of IP3 release occurred after CD3 stimulation while after CD2 stimulation there was a more sustained production of IP3 that was higher in magnitude than after CD3 stimulation. In the first 30 min, CD2 stimulation resulted in three to five-fold higher Ins(1,4,5)P3 steady state levels than after CD3 stimulation (as measured by the area under the curve of Fig. 4); this occurred even though peak calcium levels were similar after CD3 and CD2 stimulation. Thus, the prolonged nature of the calcium mobilization after CD2 stimulation was associated with prolonged production of Ins(1,4,5)P3.

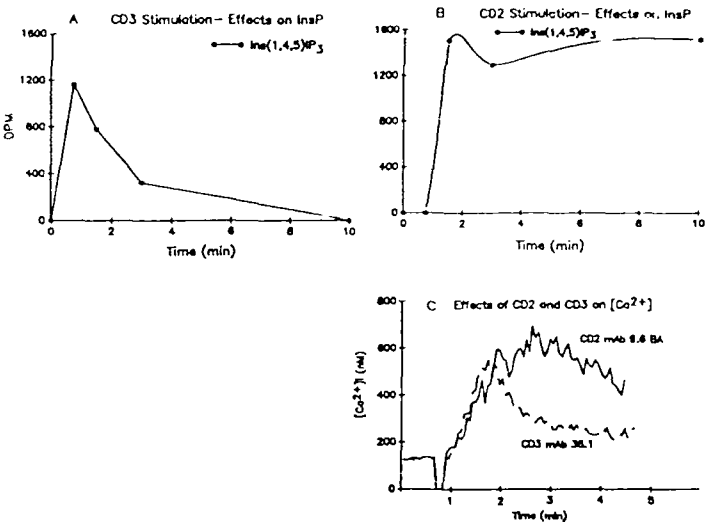


Fig. 4. Effects of CD2 and CD3 stimulation on InsP production and [Ca2+]i. T cells were isolated and loaded with [3]H-myoinositol as described for Table I. The cells were washed and stimulated with optimal amounts of CD3 mAb 38.1 (time=0) (panel A) or biotinylated CD2 antibody 9.6 (time= -5 min) followed by avidin (time= 0, panel B). Inositol phosphates were separated by FPLC, and the dpm displayed for the integrated peak of Ins(1,4,5)IP3. Simultaneous with the inositol phosphate analysis, a portion of the inositol loaded cells was loaded with indo-1 and changes in [Ca2+]i determined after CD2 and CD3 stimulation at t=0.75 min (panel C).

STUDIES ON THE ROLE OF CD4 IN LYMPHOCYTE ACTIVATION. Studies in our laboratory indicated that the crosslinking of CD4 on resting T cells could cause transient calcium mobilization that did not result in cell proliferation (12). We then found that crosslinking of CD3 and CD4 together caused a potent increase in inositol phospholipid signalling that was ½100-fold greater than could be achieved by crosslinking of CD3 or CD4 alone (29). Moreover, stimulation with soluble heteroconjugates of CD3-CD4 mAbs can induce proliferation of purified T cells and responsiveness to anti-CD28 stimulation whereas stimulation by soluble anti-CD3 alone did not result in proliferation or responsiveness to anti-CD28 (29). A number of laboratories have now published similar results that indicate CD4 has a potent role in cellular signalling through the inositol phospholipid system (30). Recent experiments have been directed at understanding what role CD4 has in the cellular signalling defect induced by HIV-1-infection. When uninfected resting or proliferating T cells were incubated with saturating amounts of CD4 mAbs for up to 48 hours the CD4 molecule was modulated off of the cell surface. Using these cells we could not reproduce the signalling defect observed after HIV infection, suggesting that modulation (internalization and phosphorylation) of CD4 is not sufficient to induce the defect. Future studies will use variants of HIV that either do or do not cause modulation of the CD4 receptor to further test this possibility (see below).

ESTABLISHMENT OF HIV-1 INFECTED LEUKEMIA T CELL LINES. The biochemical characterization of the signalling defect induced by HIV-1 infection will be facilitated by producing a cell line that exhibits the defect. To date, we have established chronic HIV-1 infection with the CEM and the Jurkat T cell leukemia lines. We have not yet characterized the Jurkat line for signalling capabilities. CEM clone A1 was infected with HIV-1 strain LAV, or with HIV-1 strain A1.9.5- 5. The A1.9.5-5 strain is a variant developed by J.A. Hoxie that causes a productive infection but does not modulate the CD4 receptor from the surface of the infected cell (see Methods for details of the infection). The cells that survived the infection had stable virus production as assessed by measurement of RT and immunofluorescence analysis of acetone-methanol fixed cells stained with HIV immune globulin (data not shown). In Table III are results of cell surface-stained CEM clone A1 cells that were analyzed 28 days after infection with LAV or with strain A1.9.5-5.

Table III

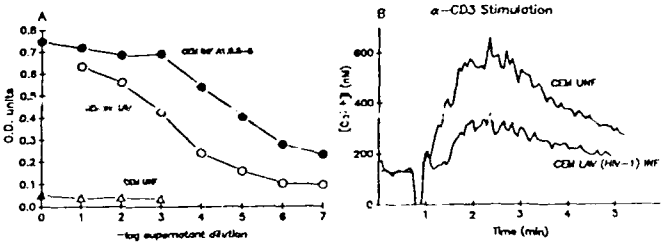
Effects of HIV-1 infection on CEM cell surface antigens

Cell Surface Antigen Expression	Cells (mean fluorescence intensity)		
	CEM UNF	CEM LAV	CEM A1.9.5-5
CD2 mAb 9.6	386	217	n.d.
CD3 mAb G19-4	162	106	82
CD4 mAb OKT4	279	43	792
CD4 mAb Leu3a	260	44	41
CD8 mAb Leu2b	46	40	n.d.
CD45 mAb 9.4	711	372	533
gp120 mAb 110.1	41	40	322
--- GAM FITC	40	40	40

Table III. CEM clone Al was infected (INF) with LAV or A1.9.5-5 and the infected or uninfected cells stained 28 days after infection. The mean fluorescence intensity (on a linear scale) was determined on 5000 cells analyzed by flow cytometry. Data were analyzed using a FACS analyzer with 4 decade log output. The gain was set to produce a mean fluorescence intensity at approximately channel 40 for control cells (stained only with GAM FITC). Values above 60 are considered positive and values above 120 are very bright.

The uninfected CEM cells were 100% CD3 and CD4 positive while cells infected with LAV had no detectable CD4 staining. It is evident that the CEM cells infected with strain A1.9.5-5 exhibited the same phenotype as observed by Hoxie et al in SupT1 cells, i.e., inhibition of anti-Leu 3 staining and near normal expression of CD4 as detected by anti-T4. In addition, the cells have easily detected constitutive viral expression as measured by an antigen capture assay (Fig. 5., panel A). When CEM clone Al was stimulated by anti-CD3 mAb, decreased calcium mobilization occurred (Fig 5 panel B); this defect was observed in cells stimulated with supra-optimal, optimal and suboptimal amounts of CD3 mAb (not shown) and occurred in spite of the preservation of the CD3 antigen as measured by cell surface expression (Table III). When the CEM clone Al was infected with the spontaneous HIV mutant A1.9.5-5, a similar signalling defect was also found (data not shown).

Fig. 5. Panel A. CEM line Al was infected (INF) with HIV-1 strain LAV, with HIV-1 strain A1.9.5-5 at an M.O.I. of 0.1 or mock infected (UNF). Infection with cytopathic effect and syncytia formation occurred that peaked on day 7, and the surviving cells were cultured and two-fold dilutions tested for HIV-1 expression on day 30 after infection by antigen capture using an ELISA assay (36). Panel B. The CEM lines were loaded with indo-1 and tested for calcium mobilization after anti-CD3 stimulation by mAb 38.1.



DISCUSSION

Previous studies from this and other laboratories indicate that the CD4 receptor has a critical role in cellular signalling; the above results indicate that the HIV-1 infection has a profound effect on calcium transients and phosphoinositol turnover after stimulation of the T cell receptor complex. These effects were found on normal lymphocytes and on leukemic T cell lines. These results provide a potential mechanism to account for the immunosuppression that is found in patients early in the course of infection by HIV-1. The contribution of the signalling defect to the overall state of immunosuppression in patients with AIDS will require further study.

A central question raised by our studies is: does HIV-1 select cells that have inherently impaired signalling capability? Alternatively, does HIV-1 itself induce a signalling deficit in cells independent of the preinfection status. There are several products of the HIV-1 genome that encode proteins that could interfere with lymphocyte signalling. The env product, gp120, appears to have the most important role in the pathogenesis of AIDS. This glycoprotein, the major component of the outer envelope of the virus, is a ligand for the CD4 receptor on T lymphocytes. Any cell that expresses CD4 appears to be capable of becoming infected with HIV. However, some cells that do not express detectable amounts of the CD4 molecule can be infected with HIV, suggesting the possibility that other mechanisms may exist to account for infection (46). There are two conserved regions in gp120, one that involves the CD4 binding site, and therefore encodes receptor function, and another that is involved in a post-binding event whereby antibodies to this region can neutralize infectivity without affecting the binding of virus to cells expressing CD4 (47,48). Of interest, it has been suggested that gp120 may directly activate CD4 cells (31).

The nef (3'orf) gene encodes a 206 residue peptide that is myristylated and associated with the cell membrane of lymphocytes (49). The function of nef is not known although it is proposed to have negative regulatory effects on viral replication because deletion of 3'orf results in viruses that have increased replication compared to wild type viruses (50,51). Recently Guy et al (52) have shown that p27, the product of nef, is phosphorylated by protein kinase C, has a GTP binding site and possesses GTPase activity. Finally the expression of nef results in down-regulation of the CD4 antigen. Thus, p27 has properties that could alter the signalling (activation) potential of HIV-1 infected cells.

Together, the current results indicate that HIV-1 infection disrupts cellular signalling at a step at or "proximal" to the activation of phosphoinositide-specific phospholipase C. Thus, one potential target of signalling disruption is that the T cell antigen receptor complex is perturbed, either directly, or indirectly by an alteration in its association with the CD4 complex. Alternatively, the effects of the virus could be at the level of the G proteins that are proposed to transmit and amplify signals from the T cell receptor to phospholipase C. Recent studies have shown that the cytoplasmic tail of the CD4 molecule is linked with a protein tyrosine kinase termed lck, a member of the src family of tyrosine kinases (53). The recent findings that both the CD3 complex (41, 54) and some of the enzymes in the phosphoinositide cycle (55) are substrates for tyrosine kinase(s) suggests a potential mechanism whereby the HIV-1 virus could cause a global impairment in cellular signalling. Our current studies are directed at determining if the effects of the virus are to disrupt the CD4-lck kinase, and thereby inhibit cellular signalling.

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