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Human immunodeficiency virus type 1 productive infection in staurosporine-blocked quiescent cells

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Staurosporine, an antibiotic known to inhibit cellular protein kinases, can reversibly block the progress of normal and tumour cells into the cell cycle. The ability of HIV-1 to infect and replicate in cells blocked by staurosporine was investigated. The results show that blocked, non-cycling cells can be productively infected by HIV-1, steadily releasing infectious progeny virus for several weeks. This suggests that at least in some cases, HIV-1 can be found in a stable and active state in resting, non-proliferating T cells.

Protein kinase inhibitor; Viral infectivity; Cell cycle; T cell

1. INTRODUCTION

Most retroviruses require that the newly infected cells undergo at least one round of DNA replication and mitosis for the synthesis of new virus to begin [1]. It is generally accepted that non-dividing T-lymphocytes cannot be productively infected by HIV-1, a retrovirus implicated in the aetiology of AIDS; only the antigen- or mitogen-activated ones can be [2]. Infection of non-activated T-lymphocytes leads to incomplete reverse transcription of HIV-RNA [3,4]. Furthermore, it has been shown that blocking the activation of lymphocytes by pre-incubation with cyclosporine A prevents them from being productively infected by HIV-1 [5].

It has been reported that binding of HIV-1 to CD4 receptor induces rapid phosphorylation of the receptor. Such phosphorylation, mediated by a protein kinase C, can be selectively inhibited by specific compounds, resulting in an accumulation of virus particles at the cell surface and inhibiting HIV infectivity at the same time [6]. Other studies have shown that protein kinase C inhibitors can exert an inhibitory effect on the entry and infectivity of several enveloped viruses [7].

Since staurosporine is a general protein kinase inhibitor, known to arrest non-transformed cells in both G_1 and G_2 phases of the cell cycle and transformed cells in the G_2 phase [8,9], it was worth investigating its effect on the ability of HIV to productively infect cell lines blocked by the presence of this antibiotic in the tissue culture medium.

Abbreviations: DMSO, dimethyl sulfoxide; HIV-1, human immunodeficiency virus type 1; IF, immunofluorescence; Mab, monoclonal antibody; PBS, phosphate-buffered saline; p.i., post-infection; TCM, tissue culture medium.

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2. MATERIALS AND METHODS

2.1. *Cells and virus*

Three human cell lines: HUT-78, a tumour T-cell line also known as H9; HSB_2, an immature T-cell line; and LDV/7, a mixed T/B cell line, were used as target cells. They were cultured in TCM: RPMI-1640 medium supplemented with 5% newborn calf serum without antibiotics, and kept at 37°C in a 5% CO_2 atmosphere. The HIV-1 virus strain used for the present study was HTLV-IIIB [10]. The virus was grown in cultures of LDV/7 cells. Supernatants of infected cultures were cleared from cells by centrifugation and filtration through 0.45 μm Millipore membranes. Such supernatants were directly used for experimental infection of fresh cells. The amount of virus present in infected culture supernatants was determined in triplicates using an ELISA kit specific for p24 major core viral protein (DuPont NEK-045, p24 cut-off: 6 pg/ml). Usually the infectious supernatants had p24 concentrations ranging from 0.4 to 1.0 ng/ml.

For viral infection, the target cells were resuspended in cell-free infectious supernatant, at a ratio of 1×10^6 cells/ml, and incubated at 37°C for 2 h. The viral inoculum was removed by centrifugation at 250×*g* and the cell pellet was resuspended in PBS and further washed twice in PBS. The final cell pellets were resuspended in TCM and transferred to Nunclon 50-ml tissue culture bottles. TCM was changed every 4 or 5 days. Cell viability of infected and control cultures was periodically assessed by Trypan blue exclusion.

2.2. *Staurosporine treatment*

Staurosporine was obtained from Sigma (St. Louis, MO, USA). Stock staurosporine was diluted in DMSO. Target cells were pre-incubated with either 70 nM (HUT-78 and HSB_2 cells) or 150 nM (LDV/7 cells) of staurosporine for six days prior to HIV infection. Viral infection and further incubation of treated cultures were carried out in presence of staurosporine at the above-mentioned concentrations. The final DMSO concentration in staurosporine-treated cultures was ≤0.1%. This concentration of DMSO has no effect on HIV replication and production by infected cells [11].

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2.3. *Virus titration*

Cell-free filtered supernatants from HIV-1 infected cultures were collected and p24 concentrations were determined by ELISA. The supernatants were further diluted with RPMI-1640 medium to obtain equal p24 concentrations in staurosporine-treated and non-treated samples. Serial 10-fold dilutions from each supernatant were made in RPMI-1640 medium.

Aliquots of each dilution (1 ml) were added to samples of fresh LDV/7 cells (about 1×10^6 cells in 1 ml of TCM without serum). The virus was allowed to adsorb at 37°C for 1 h, then the unadsorbed virus was removed by washing the cells in PBS, and the final cell pellets were resuspended in fresh TCM. Each sample was equally divided into four wells of a COSTAR Cluster^{24} tray (2 ml/well). The cultures were incubated at 37°C in 5% CO_2. On day 9 p.i., 0.5 ml aliquots from each well were collected to determine the presence of progeny virus by measuring the amounts of p24 (duplicates from each sample). $TCID_{50}$/ml was estimated by the method of Reed and Muench [12].

2.4. *Immunofluorescence*

The percentage of HIV-infected cells was determined by indirect IF using a Mab specific for p24 [11].

2.5. *Thymidine incorporation*

DNA was labelled by incubating cell cultures with methyl-[^{3}H]thymidine diluted in RPMI-1640 medium. Cultures were labelled at 0.05 μCi/ml. Triplicate aliquots (2 ml) were collected every 24 h and treated with 5% trichloroacetic acid. The acidprecipitable radioactivity was collected onto glass-fibre filters (Whatman), which were further washed with ethanol and distilled water. The radioactivity in the dried filters was counted in a toluene-based liquid scintillant.

3. RESULTS

A 72-h pre-incubation with staurosporine prevented the cells of the three cell lines from entering S phase and from further cell division, as established by the absence of [^{3}H]thymidine incorporation into cellular DNA over a period of 7 days following the addition of labelled thymidine (Fig. 1) and the lack of further increase in cell numbers in stuarosporine-treated cultures (Table I).

However, all three cell lines studied were productively infected by HIV-1 in spite of continuous incubation with staurosporine prior, during, and after the viral infection; p24 was readily detected in supernatants from staurosporine-treated cultures (Table II). At least some if not all such p24 corresponds to fully infectious HIV-1 (Table III), whilst the titers of infectious virus present in stock suspensions containing equal concentrations of p24 from either control or staurosporine-treated cultures are very much the same (Table IV). Indirect immunofluorescence, using a monoclonal antibody specific for p24, also showed that the percentage of p24$^+$ cells is quite similar in control and staurosporine-treated cultures (Table IV).

Since LDV/7 is a cell line able to sustain long-term continuous production of HIV-1 without undergoing cytopathic effects [11], it was used to study the effect of staurosporine on HIV-1 production by chronically infected cells. Table V shows that there is no clear staurosporine-induced reduction of HIV-1 production when compared with non-treated control infected cultures. LDV/7 cultures, continuously incubated in presence of staurosporine, can sustain HIV-1 production for more than 70 days, even though there is a decrease in the amount of p24 shed into the culture medium after the peak level achieved on day 14 p.i. (Fig. 2). It is worth noting that after each time-point determination, the TCM was changed by pelleting and washing the cells. Therefore, the p24 detected in each further sample is produced anew.

4. DISCUSSION

It is widely accepted that only activated lymphocytes can be productively infected by HIV-1 [2–4]. It is also

Table I

Effect of staurosporine on cell proliferation as established by cell counting

Cell line	Staurosporine (nM)	Cells/ml	
		Day 0	Day 9
HUT-78	-	3.4×10^5 (88)	2.5×10^6 (82)
HUT-78	70nM	3.4×10^5 (88)	3.7×10^5 (79)
HSB_2	-	4.2×10^5 (95)	4.4×10^6 (87)
HSB_2	70nM	4.2×10^5 (95)	2.7×10^5 (88)
LDV/7	-	2.9×10^5 (92)	4.6×10^6 (89)
LDV/7	150nM	2.9×10^5 (92)	2.3×10^5 (86)

HUT-78, HSB_2 and LDV/7 cultures were set up at the indicated initial cell densities. Direct cell counts were established every other day using a haemocytometer. The numbers in brackets represent the percentage of viable cells in each culture on the given dates. Cell viability was established by Trypan blue exclusion counting 300 cells.

Table II

Release of p24 by three human cell lines in presence of staurosporine

Cell line	Staurosporine (nM)	p24 (ng/ml)		Cell viability (%)	
		a	b	a	b
HUT-78	-	0.95	0.74	78	82
HUT-78	70	0.64	0.27	74	87
HSB_2	-	0.05	0.09	88	67
HSB_2	70	0.02	0.06	89	73
LDV/7	-	0.42	0.62	82	78
LDV/7	150	0.06	0.23	90	86

The data are from two separate experiments (a and b). Staurosporine-containing cultures were pre-incubated with the antibiotic 6 days prior to HIV infection. HIV infection and a further 8- (Expt. a) or 12-day (Expt. b) incubations were carried out in the presence of staurosporine (70 nM for HUT-78 and HSB_2 cell lines; 150 nM for LDV/7 cells). Cell-free supernatants from control and treated cultures were collected on day 8 p.i. (Expt. a) or 12 p.i. (Expt. b) and triplicate samples were assayed for p24. The S.D. among triplicates was <5%. Cell viability was established by Trypan blue exclusion counting 300 cells.

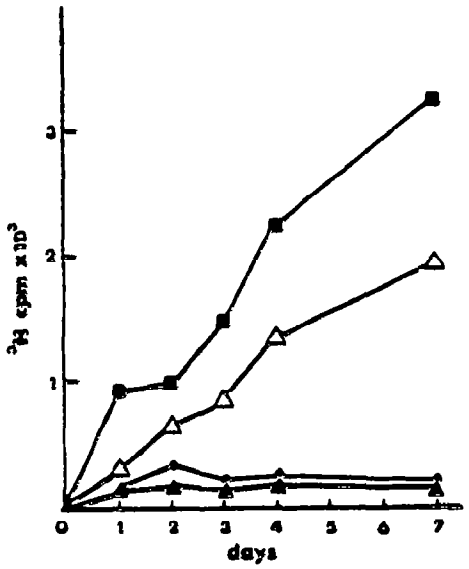

Fig. 1. Effect of staurosporine on long-term [^{3}H]thymidine incorporation into cellular DNA. HSB$_2$ cells were incubated with 70 nM staurosporine (solid triangles) from 72 h before addition of labelled thymidine, and kept in presence of staurosporine throughout the experiment. A parallel HSB$_2$ non-staurosporine-treated culture was the [^{3}H]Tdr incorporation control (open triangles). A similar experiment was performed with LDV/7 cells treated with 150 nM staurosporine (solid circles) and non-treated LDV/7 cells (solid squares). Triplicate samples from each culture were processed on the given days. The S.D. among triplicates was ≤12%.

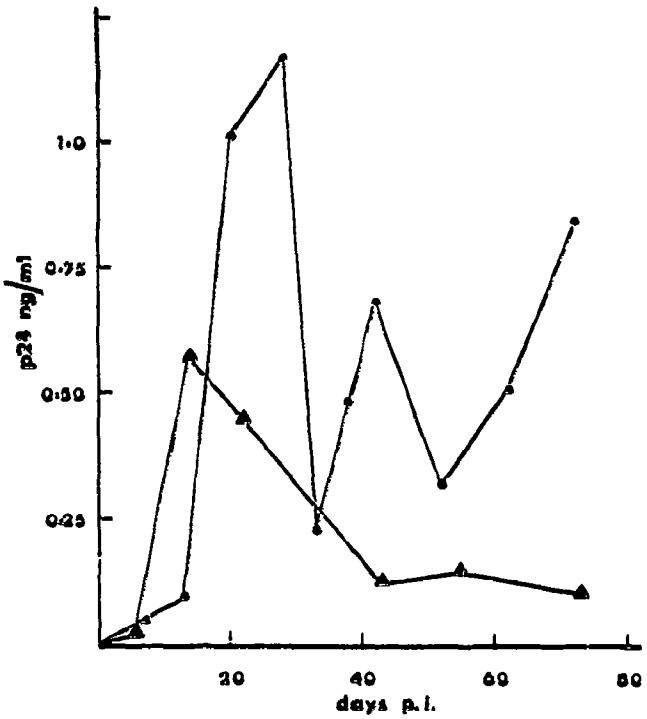

Fig. 2. Effect of continuous incubation in staurosporine on long-term HIV-1 production by LDV/7-infected cultures. LDV/7 cells were incubated with 150 nM staurosporine from day 6 before HIV infection onwards (solid triangles). A parallel non-staurosporine-treated LDV/7 culture was infected on the same day (solid circles). Presence of p24 antigen in samples (triplicates) of cell culture supernatants was determined on the indicated days post-infection. The S.D. among triplicates was ≤5%. The cell density on the day of infection was 1×10^6 cells/ml for both control and staurosporine-treated cultures. The control culture kept growing and dividing throughout the experiment. The cell density was continuously adjusted to keep it near the original value. In the staurosporine-treated culture, the cell density was continuously reduced by the lack of further cell division coupled with cell losses after each change of TCM. The cell density on day 70 p.i. was 1.35×10^6 cells/ml for the infection control and 9×10^4 cells/ml for the staurosporine-treated culture. The cell viability was 84% in the infection-control and 76% in the staurosporine-treated culture.

known that the life cycle of most retroviruses requires the growth and division of the host-cell in order to achieve stable integration of the viral genome into the host-cell DNA [1]. However, it has been reported that retroviruses of the lentivirus class, to which HIV-1 belongs, can stably replicate in non-dividing cells without the need for integration [13], and furthermore, that HIV-1 integrase-deletion mutants can readily infect T-lymphocytes, thus giving origin to extrachromosomal forms of HIV-1 DNA which are transcriptionally active, as manifested by production of p24 antigen into the culture medium. It was, however, not possible to rescue infectious virus from cultures infected with such integrase-deletion mutants [4].

Table III

Assay of infectivity of cell-free supernatants from staurosporine-treated and non-treated HIV-1-infected cultures

Supernatants from	p24 (ng/ml) in newly infected LDV/7 cultures	
	a	b
HSB$_2$	0.065	0.042
HSB$_2$ + staurosporine (70 nm)	0.062	0.037
LDV/7	0.880	0.470
LDV/7 + staurosporine (150 nM)	0.760	0.540

Cell-free supernatants from staurosporine-treated and non-treated HIV-1-infected cultures were collected on day 12 p.i. and used to infect fresh LDV/7 cells. Presence of progeny virus in the newly-infected cultures was determined on day 9 p.i. by assaying the amount of p24 present in tissue culture supernatants (triplicates); a and b indicate data from two separate experiments.

In the present study it is shown that staurosporine-blocked cells can produce newly-synthesized p24, and that some, if not all, of this p24 represents infectious HIV-1. The fact that staurosporine-treated LDV/7 cells can continuously produce HIV-1 for more than 70 days (Fig. 2) suggests that the viral DNA is in stable form within the staurosporine-treated cells. This seems consistent with recent observations which show that HIV-1 can productively infect and integrate its genome in resting monocytes [14].

The amount of p24 present in supernatants from

Table IV

Percentage of HIV-infected cells and titer of infectious virus in staurosporine-treated and non-treated HIV-1-infected cultures

Cell line	Staurosporine (nM)	p24⁺ cells (%)	Cell viability (%)	Virus titer ($TCID_{50}$/ml)
HUT-78	-	41	78	$10^{6.25}$
HUT-78	70	35	74	$10^{6.75}$
HSB_2	-	32	88	N.D.
HSB_2	70	27	89	N.D.
LDV/7	-	14	78	$10^{6.66}$
LDV/7	150	12	86	$10^{6.50}$

The percentage of p24⁺ cells were determined by IF using an anti-p24 Mab. 300 cells were counted per sample. IF was determined on days: 8 p.i. for HUT-78; 9 p.i. for HSB_2; 13 p.i. for LDV/7. Cell viability was established by Tryptan blue exclusion counting 300 cells. For virus titration, cell-free filtered supernatants from staurosporine-treated and non-treated HIV-infected HUT-78 and LDV/7 cultures were adjusted to the same p24 concentration (0.06 ng/ml), then serial ten-fold dilutions were used to infect fresh LDV/7 cells (quadruplicates). Presence of progeny virus was determined on day 9 p.i., using a p24-specific ELISA. $TCID_{50}$/ml was estimated by the method of Reed and Muench [11]. N.D.: not done.

staurosporine-treated cells is consistently smaller than that present in supernatants from non-treated cultures. However, our results suggest that the infectious-virus yield per cell is rather similar, as shown by the titration experiments, for control and staurosporine-treated cultures. Thus, the difference in p24/ml of supernatant observed between treated and non-treated cultures seems to be due to the difference in absolute cell numbers, following the arrest in cell division caused by continuous treatment with staurosporine, while in the infected control cultures the cells keep growing and dividing throughout the experiment.

It has been reported that binding of HIV-1 to CD4 receptor induces a protein kinase C-mediated phosphorylation of the receptor and that this phosphorylation is inhibited by the protein kinase C inhibitor H-7, thus resulting in accumulation of virus particles at the cell surface and subsequent inhibition of HIV infectivity [6]. However, other laboratories have failed to detect any changes in CD4 phosphorylation after binding of HIV or purified viral envelope protein gp120 [15]. Moreover, recent studies indicate that a component of the T cell receptor-CD3-dependent phosphorylation of CD4 is not mediated by protein kinase C, and that infection with HIV-1 inhibits the activation of protein kinase associated with the T cell receptor-CD3 complex by a mechanism which is independent of viral envelope proteins [15]. However, differences between the effects of H-7 and staurosporine upon cellular protein kinases have been consistently reported in the literature [16–18], and it is now well established that many staurosporine effects on cell metabolism are not due to protein kinase C inhibition [8,9,18].

The fact that staurosporine, a general protein kinase inhibitor, is unable to inhibit HIV-1 infectivity suggests that the protein kinases respectively targeted by H-7 and staurosporine are of a different nature and have a different influence in the life cycle of different viruses. Staurosporine seems to selectively inhibit a $p34^{cdc2}$ kinase-dependent mechanism that regulates the onset of cellular mitosis [8,9]. Consequently, one may infer that such a mechanism is not crucial for the life cycle of HIV-1.

The present results show that blocked non-cycling T cells can be productively infected by HIV-1. This suggests that under certain circumstances, HIV-1 can be found in a stable and active state in resting T cells. It remains to be established whether this fact may be relevant to the pathogenesis of AIDS.

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Table V

Effect of staurosporine on HIV-1 production by chronically infected LDV/7 cells

Days post-treatment	p24 (ng/ml)	
	In presence of staurosporine	In absence of staurosporine
0	1.50	1.36
7	1.25	0.42
15	0.80	0.64

LDV/7 cultures infected with HIV-1 for 29 days were transferred to fresh TCM with (150 nM) or without staurosporine and further incubated for 15 days. The amounts of p24 in cell-free supernatants (triplicates) were determined on the indicated days post-treatment. The S.D. among triplicates was ≤5%.

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