HIV-induced cytopathology and viral load in a pentamidine-treated lymphocytic cell line

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Preincubation of CD4 lymphocytes with pentamidine isethionate at a concentration of 1.5 mg/L then removal from incubation medium prior to addition of HIV-1, or incubation of cells with the drug and virus simultaneously, increased HIV-1 DNA load but reduced p24 antigen release. The number of syncytia generated was not affected by the presence of pentamidine. The extent of balloon degeneration of cells was greater, however, although this was not associated with a discernable increase in cellular necrosis or reduction in cell viability. This suggests that drug-treatment resulted in an increased load of intracytoplasmic (but not necessarily integrated) forms of HIV-1; this may explain the lower levels of antigen produced and also the balloon degeneration of treated cells, a phenomenon previously observed with other retroviruses.

Introduction

The isethionate salt of the aromatic bis (amidine) pentamidine is a relatively effective, though reportedly toxic drug when used for the treatment and prophylaxis of Pneumocystis carinii pneumonia (PCP) in individuals with AIDS (Wispelwayne & Pearson, 1991). When administered parenterally, the drug has a therapeutic role in the treatment of the leishmaniasis and pre-CNS African trypanosomiasis (Sands, Kron & Brown, 1985). Nebulized pentamidine may also be useful as a local agent for prophylaxis of intra-oral candidosis in HIV-infected individuals (Nolan et al., 1994).

It has been reported that pentamidine and certain other closely related compounds (eg stilbamidine isethionate and diminazene aceturate) exhibit a mild, structure-dependent inhibitory effect against the oncornaviral DNA polymerase (reverse transcriptase) of Moloney murine leukemia virus (De Clercq & Dann, 1980).

In view of this latter observation the present investigation aimed at determining the effect of pentamidine isethionate on the interaction of HIV with an established laboratory lymphocyte-cell line. Specifically, the effect of a therapeutic concentration of the drug on total viral load (ie integrated provirus and intracytoplasmic forms) of host lymphocytes, p24 (core) viral antigen production within these cells, formation of syncytia (Dowsett et al., 1987) and infectious virus production, were examined.
Materials and methods

**Lymphocytes**

A human-derived T-lymphoblastoid cell line (C8166, MRC Potters Bar) was maintained in RPMI 1640 medium (Gibco) containing 1% 100 mM sodium pyruvate, 1% 200 mM L-glutamine and 10% fetal calf serum (all reagents from Gibco). Penicillin (100 IU/mL), streptomycin (100 IU/mL) and amphotericin B (1.25 mg/L) were included in the medium in order to control bacterial and fungal contaminants respectively.

Lymphocyte cultures were routinely incubated at 37°C in a 5% CO₂:95% air mixture. After 8 days fresh culture medium was added. Cell viability was routinely assessed using a standard trypan blue exclusion test (Hudson & Hay, 1989). Only cohorts of cells having ≥98% viability were used.

**HIV propagation**

The syncytium-forming RF strain of HIV-1 (Popovic et al., 1984) was maintained by continuous culture in the above system.

**Drug**

Pentamidine isethionate (Sigma) was incorporated into RPMI 1640 medium at a concentration of 1.5 mg/L. The latter is the upper limit detectable in plasma after a single parenteral dose of 4 mg of the isethionate salt (Arnott Hay & Croft, 1988) per kilogram body weight (Waalkes & DeVita, 1971).

**Lymphocyte treatments**

Treatment 1 comprised lymphocytes (4 × 10⁵ cells) suspended in 10 mL RPMI 1640 medium containing pentamidine at a concentration of 1.5 mg/L. Treatment 2 comprised lymphocytes (4 × 10⁵ cells) preincubated in 10 mL RPMI medium containing 1.5 mg/L of drug. After 8 days, these cells were washed with medium to remove residual drug prior to viral infection. This was performed to determine if intracellular pentamidine (Arnott et al., 1991) affected the HIV-1-lymphocyte interaction. Treatment 3 (positive control) comprised lymphocytes (4 × 10⁵ cells) suspended in 10 mL drug-free RPMI 1640. Pre-titrated cell-free virus harvested from the supernate of an infected culture was introduced into cultures from each treatment group at a 0.5 multiplicity of infection. Treatment 4 (negative control) comprised lymphocytes (4 × 10⁵ cells) exposed to neither drug nor virus. Six replicates of each of the four treatments were incubated in 25 cm² tissue culture flasks (Costar) at 37°C in an atmosphere of 5% CO₂:95% air for 8 days.

**Total viral DNA content of lymphocytes**

C8166 cells from each of the treatments were suspended in 400 μL of lysis buffer (0.05 mM EDTA, pH 8.0; 0.1 mM Tris-HCl pH 7.5; 50 mM NaCl) containing 4 μL of proteinase K (Boehringer Mannheim, 10 g/L), 44 μL of 10% Sarkosyl (Sigma) and incubated at 65°C for 2 h. Total intracellular DNA was extracted twice in phenol and once in chloroform prior to precipitation (overnight in ice-cold 100% ethanol), centrifugation (20 min at
1500 rpm) and vacuum drying. Purified DNA was resuspended in 100 μL DEPC treated water.

A nested PCR method (Simmonds et al., 1990; Williams et al., 1990) was used for detection, after amplification of the pol region of the genome, of both integrated proviral and unintegrated intracytoplasmic forms of HIV-1. Subsequent quantification of viral DNA was achieved using a limiting dilution method.

*p24 antigen production*

HIV-1 core antigen production was assessed using a commercial sandwich ELISA (Coulter, Hialeah, Florida, USA).

*Syncytium formation*

The number of syncytia present in each treatment after 8 days of incubation was scored using an inverted microscope: 10 syncytia per field: ++ +; 3–9: ++; 1–2: +. The morphological appearance of syncytia was also noted.

*Infectious virus titration*

Suspensions of C8166 cells (0.1 mL containing 2 x 10⁵ cells) were added to wells of flat-bottomed microtitre plates (96 well, Costar). An equal volume of a serial 10-fold dilution series (1–10⁸) of supernates from each treatment was added to the appropriately assigned well. Six replicates of each dilution series from each test supernate was performed. Plates were sealed and incubated for 1 h at 4°C; this standardised both viral attachment and entry into lymphocytes. Excess medium was removed and wells gently flooded with fresh medium in order to remove extracellular virus and/or virus particles. After removal of the medium, cells containing intracellular HIV-1 were resuspended in 0.5 mL of RPMI medium for 8 days at 37°C in an atmosphere of 5% CO₂:95% air. The number and appearance of syncytia in each well was noted. Virus titrations of treatment supernates (TCID₅₀) were calculated using the method of Reed & Muench (1938).

**Results**

*Total viral DNA content of lymphocytes*

Negative control cultures did not contain HIV-1 DNA using the nested PCR method. The Table shows that cells preincubated or incubated with pentamidine contained more viral copies per cell (by a factor of 10) than non-drug treated cells; there was no difference between these levels in cells which had been preincubated or incubated with the drug. There was, therefore, more viral DNA present within the pentamidine-treated cells.

*p24 antigen production*

Supernates harvested from uninfected cultures did not contain p24 antigen as measured by ELISA.
The Table indicates that supernates harvested from cultures incubated with pentamidine contained reduced levels of p24 antigen compared to non-drug treated controls. Supernates from cultures preincubated with pentamidine contained less antigen than supernates from cultures incubated with the drug.

**Syncytium formation**

In the absence of HIV-1, lymphocytes showed no syncytium formation. The number of syncytia formed in HIV-1 cultured cells did not differ when pentamidine was present, and displayed marked ballooning of plasma membranes. Absence of cellular debris in these cultures indicated that cell necrosis was not a feature. Trypan blue exclusion showed that cells remained viable whilst concurrently displaying this cytopathic effect.

**Infectious virus titration**

The TCID$_{50}$ of supernates harvested from cultures preincubated with pentamidine was decreased relative to cultures not exposed to drug; that from cultures incubated with pentamidine was not reduced within the limits of the test. Preincubation with pentamidine was associated with a greater reduction relative to incubation, in the amount of virus released. The latter trend was similar to that of the p24 antigen assay.

**Discussion**

Findings from the present study indicate that pentamidine affects certain elements in the interaction between HIV-1 and CD4 lymphocytes *in vitro*. There was no evidence, however, for pentamidine induced effects on reverse transcriptase, either directly or indirectly, as observed (De Clercq & Dann, 1980) with the oncornavirus Moloney murine leukemia virus.

| Table. Effect of pentamidine isethionate on the HIV-1-lymphocyte interaction |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | C8166 cells without HIV     | C8166 cells with HIV         |                             |
|                             | No drug treatment           | Incubated with drug*         | Preincubated with drug*      |
| Cells per viral copy*       | ND                          | 15                          | 1.5                         | 1.5                         |
| Total viral copies per cell*| ND                          | 0.066                       | 0.66                        | 0.66                        |
| p24 antigen per cell (pg)*  | 0                           | 1.14                        | 0.13                        | 0.05                        |
|                             | (0.9–1.17)                  | (0.05–0.22)                 | (0.04–0.12)                 |
| Syncytium formation*        | —                           | ++                          | ++                          | ++                          |
| TCID$_{50}$*                | 0                           | $10^5$                      | $10^{3.56}$                 | $10^{5.56}$                 |

*Incubation: pentamidine and HIV-1 present during test period.
*Preincubation: cells exposed to pentamidine prior to infection.
*For HIV treatments, the greater the number of cells recorded, the lower the level of infection.
*Reciprocal of cells per viral copy.
*Immunocytochemistry confirmed this trend (unpublished data).
*Number of syncytia formed in each treatment were identical; but balloon degeneration greater with drug-treatment.
*50% end point of tissue culture infective dose.
The study aimed at assessing the effect of pentamidine on HIV-1 infection and replication within CD4 lymphoblastoid cells. Two treatment modalities were chosen: first, preincubation of lymphocytes with pentamidine in the absence of HIV-1 after cellular uptake of drug, then removal of its presence extracellularly; and, secondly, incubation of cells, HIV-1 and drug simultaneously. The former was included since pentamidine is known to locate intracellularly when incubated, for example, with human neutrophilic granulocytes (Arnott et al., 1991). Both procedures aimed at determining whether pentamidine had a direct effect on the ability of the virus to infect the host cell.

Pentamidine reduces the ability of human neutrophilic granulocytes to kill *Candida albicans* spores in vitro (Arnott & Hay, 1989a), and also affects deleteriously other functions of the phagocytic cells (Arnott & Hay, 1989b). The drug may have some role in prophylaxis of candidosis in AIDS patients (Nolan et al., 1994), but it is unlikely that the effect is direct, since the drug is considered to be ineffective against yeasts and other fungi (Christison & Conant, 1953).

Pentamidine can influence membrane-associated functions of cells including the NADPH dependent oxidase system of human neutrophilic granulocytes at least in vitro (Arnott & Hay, 1990). This is, however, but one of the proposed mechanisms by which pentamidine can act on target cells at the molecular level (Kapusnick & Mills, 1988). Pentamidine (and related compounds diminazene aceturate and hydroxystilbamidine) induces synchronous differentiation from the active trophozoite to dormant cyst form in the free-living protozoan *Acanthamoeba*, a process which may be due to irreversible inhibition of the enzyme SAMDC (Gupta, Shukla & Walter, 1987) a key enzyme in the polyamine biosynthetic pathway. This effect also occurs in mammalian cells (Karvonen et al., 1985) and in trypanosomes (Bitonti, Dumont & McCann, 1986). The effect of pentamidine on such metabolic pathways of HIV infected lymphocytes and the host cell-HIV interaction cannot be assessed from findings for the present study, but clearly merits further investigation.

Pentamidine is also known to interact directly with nucleic acids interfering with the incorporation of nucleotides into RNA and DNA. This is thought to be central to the mechanism by which the drug acts at the molecular level. Studies involving X-ray crystallography and atomic absorption (Edwards, Jenkins & Weidle, 1992), 2-D NMR spectroscopy (Jenkins et al., 1993), footprinting (Fox, Sansom & Stevens et al., 1990) and molecular modelling analysis (Sansom et al., 1990) collectively indicate that pentamidine binds preferentially to the minor groove DNA at AT rich sequences. This occurs in a manner analogous to that of the antiviral agent netropsin and the structurally related anti-trypanosomal drug diminazene aceturate or Berenil (Bailly et al., 1994). No unequivocal evidence is available, however, to show that integration of HIV into the host cell genome is other than random (Levy, 1983).

In the present study, there was a ten-fold increase in viral load (i.e. the amount of HIV-1 DNA present in the host cell), when pentamidine was involved in the virus-host cell interaction. The amount of p24 (core) antigen released from HIV-1 infected lymphocytes into the culture medium was diminished, however, when cells were/had been exposed to pentamidine. The latter effect was noticeably greater with cells which had been preincubated with the drug. The greater ballooning of pentamidine-treated cells during syncytium formation, taken together with the corresponding decrease in p24 antigen release suggests that pentamidine renders the intra-lymphocytic HIV-1 infection
relatively non-productive, although there is a greater load of viral DNA within the lymphocytes.

HIV-1 proviral DNA integrated into the host cell genome or intracytoplasmic DNA forms of HIV-1 (Levy et al., 1985; Shaw et al., 1985) which may have been present within the host cells were not differentiable using methods applied in this study. Accumulation of such intracytoplasmic virus is known with infections of, for example, avian leukosis virus and spleen necrosis virus, to lead to cell necrosis (Keshet, O'Rear & Temin, 1979; Weller & Temin, 1981; Chen & Temin, 1982; Hoover et al., 1987). If the DNA is fully integrated within the host cell genome, and is not located in the cytoplasm, it represents a large potential reservoir of virus which is not reflected in either p24 antigen production or infectious virus yield. If this is the case, then pentamidine may be associated with increased retention of the virus within lymphocytes. Virus titration showed that at least some virus released from infected lymphocytes was viable; this may indicate that pentamidine is not directly virucidal.

Syncytium formation induced by viral envelope proteins ultimately leads to cell necrosis; the precise mechanism(s) remains unresolved. The process facilitates penetration of HIV into CD4 cells as a consequence of insult to their plasma membrane (Fermin & Garry, 1992). Cells exhibiting cytopathic effects due to HIV are unable to control the influx of monovalent and divalent cations and these accumulate within the cell (Cloyd & Lynn, 1991). This can occur in certain neurological disorders including HIV associated dementia, where inappropriate glutamate receptor activation, particularly the NMDA, can lead to neuronal death (Reynolds et al., 1993). The process occurs as a consequence of calcium ion entry into the cells and it is known that this cation may be cytotoxic under certain conditions (MacDermott et al., 1986; Choi, 1987; Murphy, Thayer, & Miller, 1987).

These events do not provide a complete explanation for the findings from the present study. Neither can accumulation within cells of non-integrated HIV DNA, a situation which has also been implicated as a cause of cell necrosis (Levy et al., 1985; Shaw et al., 1985). Unexpectedly premature cell necrosis was not a feature of any of the drug-virus-cell interactions investigated in the present study.

The stage of the HIV infection process in vivo at the time of sampling must be considered when isolation of the virus from clinical samples is attempted. Isolates (slow/low variants, Simmonds et al., 1991) from HIV-1 infected individuals taken from the early stage of infection (asymptomatic) are often non-cytopathic; they exhibit suboptimal growth in human donor peripheral blood mononuclear cells (PBMCs); such viral isolates are not supported in the T-cell line culture system used in this study (Asjo et al., 1986; Cheng-Mayer et al., 1988; Tersmette et al., 1988). When the clinical symptoms of the infection are more severe, however, the virus may be more readily isolated from PBMC and T-cell line culture.

Individuals infected with such a non-cytopathic slow/low variant of HIV-1 who are concurrently undergoing prophylaxis with pentamidine for PCP, may experience trends similar to those observed in the present study but HIV may be undetectable by infectivity assays. Thus, in the present study it was considered appropriate to use the RF strain of HIV-1 which is highly cytopathic, and to utilise an adapted neoplastic T-cell line. This, it was considered, would provide optimal conditions for assessment of the possible effects of pentamidine on the virus-cell interaction.

It would be advantageous to investigate the effect of pentamidine treatment on PBMCs infected with either the RF strain of HIV-1 or clinical isolates from various
stages of the disease process. Further, the findings from the present study do not include a consideration of immunological modulation in the drug-HIV-lymphocyte interaction in vivo. The effect of pentamidine on cells of the immune system, however, should be a cause of considerable concern in clinical medicine in view of the findings from the present study.

References


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