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DECREASED ANTI-HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 ACTIVITIES OF 2', 3'-DIDEOXYNUCLEOSIDE ANALOGS IN MOLT-4 CELL SUBLINES RESISTANT TO 2', 3'-DIDEOXYNUCLEOSIDE ANALOGS

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Summary. – Human T-lymphoid MOLT-4 cells were grown continuously for more than 1 year in medium containing either 3'-azido-2', 3'-dideoxythymidine (AZT), 2', 3'-dideoxyinosine (ddI) or 2', 3'-dideoxycytidine (ddC) at concentrations similar to peak plasma levels found in clinical trials in patients with AIDS. To test antiviral activities of the nucleoside analogs against HIV-1 in the cell sublines designated MOLT-4^r-AZT, MOLT-4^r-ddI and MOLT-4^r-ddC, the number of infected cells, p24 HIV-1 antigen in culture medium and syncytium formation of infected cultures were determined. The results showed that anti-HIV-1 activities of AZT, ddI and ddC were significantly decreased in the resistant MOLT-4 cell sublines grown continuously with the respective nucleoside analog, probably due to the development of cell populations resistant to the drugs.

Key words: nucleoside analogs; drug resistance; HIV; AIDS

Introduction

Failure of anti-human immunodeficiency virus (HIV) chemotherapy using AZT was observed in patients with acquired immunodeficiency syndrome (AIDS) who underwent prolonged therapy with AZT (Fischl *et al.*, 1989; Richman *et al.*, 1990). A decreased sensitivity to AZT together with multiple point mutations in the *pol* gene were demonstrated in HIV isolates obtained from such patients (Larder *et al.*, 1989*a, b*; Land *et al.*, 1990; Japour *et al.*, 1991; Boucher *et al.*, 1992). Similarly, clinical HIV-isolates with decreased sensitivity to ddI or ddC were obtained from patients with AIDS treated for prolonged periods with ddI or ddC, respectively (St Clair *et al.*, 1991; Howell *et al.*, 1991). For these reasons, it seems probable that the development of a resistant virus population is a relevant factor in clinical resistance. On the other hand, lack of clinical response does not always mean that the isolate is resistant (Larder *et al.*,

1989*a*; Land *et al.*, 1990) and mechanisms other than virus resistance must be assumed to account for the failure of antiviral chemotherapy (Swierkosz, 1992).

We suppose that cell resistance to antiviral agents can evolve during the prolonged chemotherapy especially in patients with AIDS. Consequently, the resistant cells would contain subtherapeutic levels of antiviral agents or of their active forms resulting in therapeutic failure. Cell resistance to different chemical agents including nucleoside analogs was shown to be the main obstacle in cancer chemotherapy (Henderson, 1984; Young, 1989). A variety of cell lines which developed *in vitro* resistance to chemical agents were useful for the study of resistance mechanisms of cancer cells (Beck and Danks, 1991). Previously, we have shown that in a monkey kidney cell line the development of a cell population resistant to acyclovir results in decreased antiviral effects of acyclovir in these cells against herpes simplex virus type 1 (Cinatl Jr. *et al.*, 1992*a*). In a preliminary communication we reported that in MOLT-4 cells grown for 10 months in medium containing ddC its anti-HIV-1 effects were significantly decreased (Cinatl *et al.*, 1992). In this study, we present results dealing with anti-HIV-1 activities of AZT, ddI and ddC in MOLT-4 cell sublines grown for more than 1 year in medium containing the respective nucleoside analog.

Materials and Methods

Cells and viruses. All culture media and foetal bovine serum (FBS) were purchased from Seromed (Berlin, Germany). MOLT-4 cell line was a generous gift from Dr. Löwer (Paul-Ehrlich-Institute, Langen, Germany). All cells were propagated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10 % FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were routinely tested for mycoplasma by the Hoechst 33258 DNA staining method (Chen *et al.*, 1977) and found to be free of contamination. The source of HIV-1 were chronically infected H9/HTLV-III$_B$ cells propagated continuously in protein-free medium (Cinatl Jr. *et al.*, 1992*b*). Supernatants of these cells were stored at -70 °C and titrated for infectious virus ($TCID_{50}$) in MOLT-4 cells.

Drugs. AZT, ddI and ddC were obtained from Sigma. All nucleoside analogs were dissolved in dimethylsulphoxide. Stock solutions were stored at -20 °C.

Cytotoxicity assay. The cells were cultured with and without drugs for 5 days at which time aliquots were counted for cell viability using haemocytometer. Viability of the cells was determined by the dye exclusion method after staining with 0.5 % trypan blue solution. Cytotoxicity of a drug was expressed in CC_{50}, a concentration reducing the number of viable cells by 50 %.

Antiviral activity assay. Cells were infected with HIV-1/HTLV-III$_B$ at multiplicity of 0.1 $TCID_{50}$/cell and incubated without or with different concentrations of the drugs. The antiviral affects were assessed 6 days p. i. The amounts of HIV-1 p24 antigen in culture supernatants were measured using commercial p24 antigen ELISA kits (Abbott). The method was performed according to the manufacturer's instructions. The infected cells were examined for the presence of HIV-1 by the immunoenzymatical alkaline phosphatase-anti alkaline phosphatase (APAAP) method using monoclonal antibodies against HIV-1 p24 core protein (Dianova, Hamburg, Germany). The tests were performed using assay kits (Dianova) in accordance with the manufacturer's instructions. The antiviral activity of a drug was expressed in EC_{50} and EC_{90}, respectively, representing concentrations reducing the amount of p24 antigen or the number of infected cells by 50 % and 90 %, respectively.

Results

To expose MOLT-4 cells to nucleoside analogs at concentrations similar to peak plasma levels found in patients with AIDS (Yarchoan *et al.*, 1989), the cells were grown in medium with AZT, ddI and ddC at a concentration of 5, 10 or 0.5 μmol/l, respectively. MOLT-4 cells were directly seeded in medium containing either AZT or ddI at the respective concentration without any significant decrease in growth rate in successive passages. In contrast, MOLT-4 cells died after 3 subcultures in medium containing 0.5 μmol/l ddC. To avoid delayed toxic effects of ddC in successive passages, MOLT-4 cells were grown for 5 subcultures in medium containing 0.1 μmol/l ddC and for 6 subcultures in medium containing 0.2 μmol/l ddC, before being propagated in medium

Table 1. Cytotoxic effects of AZT, ddI and ddC on MOLT-4 and MOLT-4^r cells

Drug	CC_{50} (μmol/l)			
	MOLT-4	MOLT-4^r-AZT	MOLT-4^r-ddI	MOLT-4^r-ddC
AZT	52 ± 4.9	480 ± 51	ND	ND
ddI	850 ± 41	ND	>1000	ND
ddC	16 ± 2.2	ND	ND	250 ± 14

The results were obtained from three independent experiments. In each experiment cells were counted in triplicate for each drug concentration and values are mean ± standard error. ND = not done.

Table 2. HIV-1 titers in resistant MOLT-4^r sublines

Cells	$TCID_{50}$/ml		
	Number of subcultures of resistant cells in medium without the nucleoside analogs		
	0	3	5
MOLT-4^r-AZT	2.1×10^2	4.1×10^4	6.5×10^5
MOLT-4^r-ddI	1.5×10^1	3.9×10^3	4.9×10^5
MOLT-4^r-ddC	2.9×10^2	3.2×10^3	5.1×10^5

The cells were maintained after infection in medium without the nucleoside analogs. Viral titers in normal MOLT-4 cells ranged from 4.1×10^5 to 9.3×10^5 $TCID_{50}$/ml.

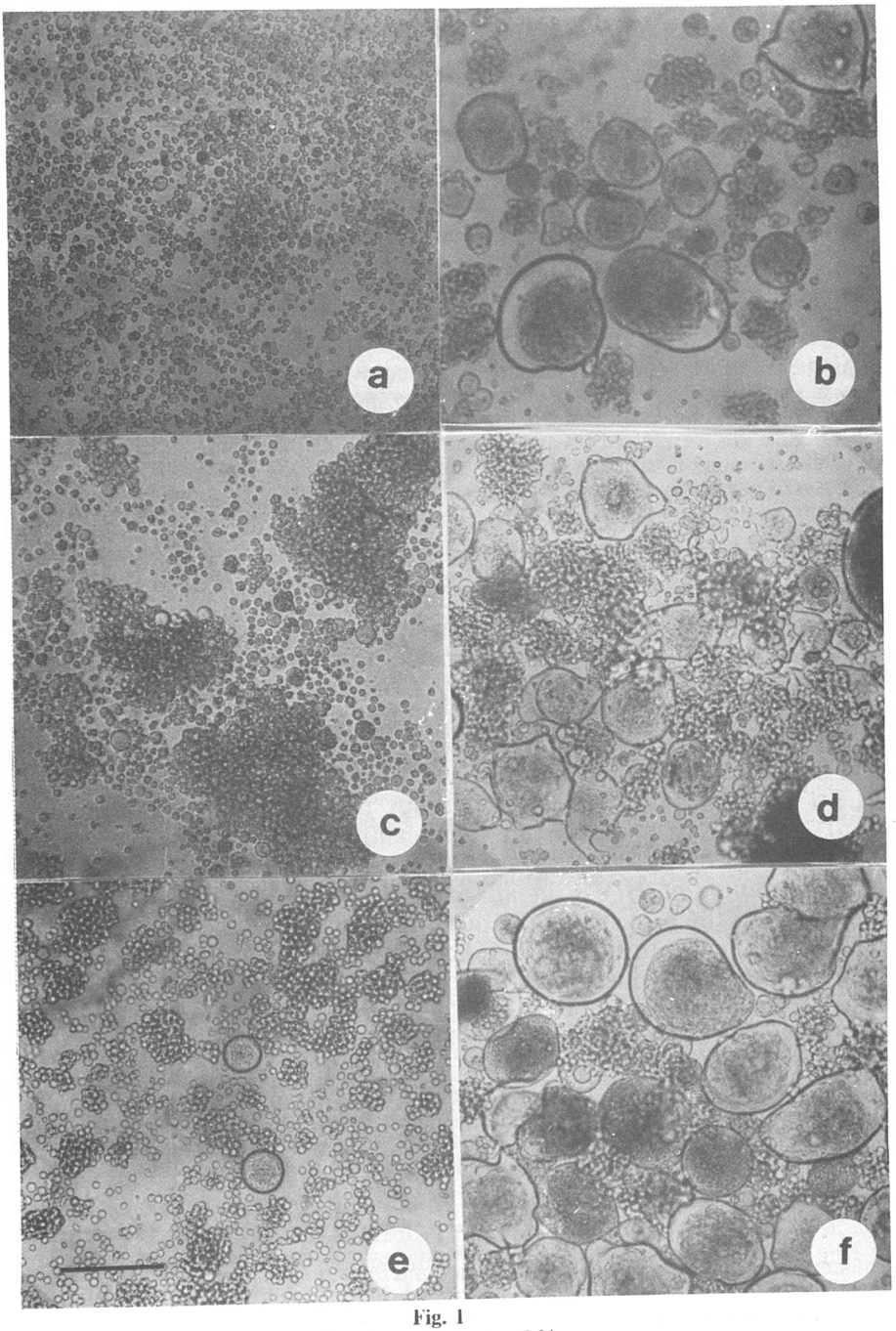

Fig. 1
For legend see page 364

Table 3. Antiviral activity of AZT in MOLT-4 and MOLT-4^r-AZT cells

Cells	ELISA		APAAP	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}
MOLT-4	0.02 ± 0.001	0.26 ± 0.01	0.03 ± 0.006	0.19 ± 0.008
MOLT-4^{r}AZT	0.39 ± 0.02	33 ± 2.9	0.51 ± 0.08	29 ± 3.2

ELISA. The results were obtained from three independent experiments. In each experiment values (mean ± standard error) were obtained from at least two dose response curves.
APAAP. The results were obtained from two independent experiments. In each experiment the number of cells which expressed p24 antigen were determined in triplicate for each drug concentration. Values are mean ± standard error.
MOLT-4^r-AZT cell sublines were used after 5 subcultures (30 days) in medium without nucleoside analogs.

containing 0.5 μmol/l ddC. MOLT-4 sublines (designated by an 'r' superscript and the nucleoside analog used for selection) were subcultured at six day intervals at a ratio 1:5 and the nucleoside analogs were added to a culture medium at two day intervals. MOLT-4^r-AZT and MOLT-4^r-ddI after 15 months (77 subcultures) and MOLT-4^r-ddC after 13 months (67 subcultures) in medium with the respective nucleoside analog were used in these experiments.

The proliferation of MOLT-4^r sublines did not differ markedly from that of MOLT-4 cells grown in medium without nucleoside analogs (results not shown). However, CC_{50} values were significantly higher in all resistant MOLT-4^r sublines than in normally propagated MOLT-4 cells (Table 1).

The replication of HIV-1 in all resistant MOLT-4 cells sublines grown continuously in the presence of the nucleoside analogs was significantly impaired (Table 2). In contrast, after 5 subcultures (30 days) of MOLT-4^r cells in medium without the nucleoside analogs similar viral titers and number of infected cells were found both in MOLT-4^r and MOLT-4 cells. Therefore, to assess the activity of the nucleoside analogs against HIV-1, MOLT-4^r cells grown for at least 5 subcultures without the drug were used. The EC_{50} and EC_{90} values were significantly higher in all MOLT-4^r cell sublines than in MOLT-4 cell line (Tables 3, 4 and 5). In addition, higher concentrations of the nucleoside analogs were required to inhibit cytopathic effects in MOLT-4^r cell sublines than in normally propagated cells (Fig. 1). The decreased antiviral affects of AZT, ddI

Fig. 1

Inhibitory effect of nucleoside analogs on syncytium formation in sublines of MOLT-4 cells
0.2 μmol/l of AZT in MOLT-4 (a) and MOLT-4^r-AZT (b) cells;
0.5 μmol/l of ddI in MOLT-4 (c) and MOLT-4^r-ddI (d) cells;
10 μmol/l of ddC in MOLT-4 (e) and MOLT-4^r-ddC (f) cells. Bar = 200 μm.

Table 4. Antiviral activity of ddI in MOLT-4 and MOLT-4^r-ddI cells

Cells	ELISA		APAAP	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}
MOLT-4	3.8 ± 0.4	21 ± 1.6	4.6 ± 0.6	27 ± 1.9
MOLT-4^r-ddI	35.39 ± 1.18	195 ± 12	39.51 ± 1.9	230 ± 19

ELISA, APAAP: see legend to Table 3.
MOLT-4^r-ddI cell sublines were used after 5 subcultures (30 days) in medium without nucleoside analogs.

Table 5. Antiviral activity of ddC in MOLT-4 and MOLT-4^r-ddC cells

Cells	ELISA		APAAP	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}
MOLT-4	3.8 ± 0.4	21 ± 1.6	4.6 ± 0.6	27 ± 1.9
MOLT-4^r-ddC	35.39 ± 1.18	195 ± 12	39.51 ± 1.9	230 ± 19

ELISA, APAAP: see legend to Table 3.
MOLT-4^r-ddC cell sublines were used after 5 subcultures (30 days) in medium without nucleoside analogs.

and ddC were also observed in MOLT-4^r cell sublines grown for 3 months (15 subcultures) in medium without the nucleoside analogs (results not shown).

Discussion

The failure of AZT, ddC or ddI to inhibit efficiently HIV-1 replication in MOLT-4^r cell sublines is believed to result from the development of resistant cell populations. The low cytotoxic effects of the drugs in MOLT4^r cells strongly support this assumption. The 2', 3'-dideoxynucleoside analogs are phosphorylated intracellulary by cellular nucleoside kinases to their triphosphates before acting as cytotoxic or antiviral agents (Cooney *et al.*, 1986; Furman *et al.*, 1986; Starnes and Cheng, 1987; Balzarini *et al.*, 1989; Johnson and Fridland, 1989). The resistance of MOLT-4^r cells to the cytotoxic effects of the drugs and their higher doses required to inhibit HIV-1, could well be due to sublethal or subtherapeutic levels of the triphosphates of the nucleoside analogs within the resistant cells. It has been demonstrated that deficiency in deoxycytidine kinase

may account for decreased anti-HIV activity of some dideoxynucleoside analogs in cultured T lymphoblast cells (Haertle *et al.*, 1988). In addition to decreased activity of cellular nucleoside kinases other mechanisms known to be responsible for the resistance of tumour cells to nucleoside analogs such as defective transport, elevated levels of degradative enzymes and altered intracellular nucleotide pools (Henderson, 1984; Young, 1989), could also evolve in MOLT-4^r cells sublines. In our laboratory, studies are in progress to compare uptake and metabolism of the dideoxynucleoside analogs in MOLT-4 and MOLT-4^r sublines.

An interesting possibility is raised by the presence of multidrug resistance, i. e. resistance of cells to multiple, structurally unrelated chemotherapeutic agents (Biedler and Riem, 1970). The cells with multidrug resistance express a 170 K membrane glycoprotein (p170), which acts as an energy dependent efflux pump responsible for decreased drug accumulation in the cells (Ling and Thompson, 1974; Chen *et al.*, 1986; Fojo *et al.*, 1987). In cultures of H9 and U937 cells, p170 was shown to account for a decreased accumulation of AZT in the cells infected with HIV-1 (Gollapudi and Gupta, 1990). Importantly, p170 expression was induced directly by HIV-1 infection without previous treatment of the cells with AZT. Multidrug-resistant CEM VBL 100 cels, which express high levels of P-glycoprotein, were shown to be less sensitive to both the antiproliferative activity and the antiviral action of AZT (Antonelli *et al.*, 1992). However, in our tests, we were not able to find p170 in any MOLT-4^r cell sublines using immunocytochemical methods (results not shown).

It is not clear whether cell resistance to antiviral agents is a relevant factor in clinical resistance. However, the lack of clinical response to nucleoside analogs does not always mean that patients harbour a resistant virus population; and the development of a resistant cell population provides possible explanation. Notably, cell resistance to different chemical agents does not develop only in cancer cells but may also occur in normal cell populations (Schimke, 1986; Fojo *et al.*, 1987). Moreover, gene mutation and the selection of a resistant cell population are not necessarily required, since factors which account for a resistant phenotype may be regulated at the level of gene transcription (Chin *et al.*, 1992). These observations strongly encourage studies to show whether cell resistance to antiviral agents is of importance in patients with viral diseases. Positive findings would significantly influence strategies in antiviral chemotherapy.

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