RIDOSTIN INHIBITS HIV-1 REPLICATION IN THE T LYMPHOBLASTOID CELL LINE C8166. POSSIBLE ROLE OF ALTERED CYTOKINE PRODUCTION

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Received March 13, 1995; revised June 6, 1995

Summary. – Altered cytokine production in human immunodeficiency virus 1 (HIV-1) infection is well documented and cytokine modulators are currently under investigation as possible therapeutic agents. We tested the ability of Ridostin (dsRNA preparation derived from *S. cervisiae*) to inhibit HIV-1 replication in actuely infected T lymphoblastoid C8166 cells. Ridostin inhibited HIV-1 replication in a concentration range that is 100-fold lower than the toxic concentration for these cells. C8166 cells spontaneously produced interferon (IFN) alpha and gamma, as well as tumor necrosis factor (TNF) alpha. Ridostin activated IFN alpha and suppressed TNF alpha and IFN gamma production by these cells. Monoclonal antibodies (MoAbs) to TNF alpha dose-dependently inhibited HIV-1 replication in these cells. Therefore it is possible that the observed anti-HIV activity of Ridostin in C8166 cells is partly mediated by altered cytokine production. Particularly, suppression of TNF alpha synthesis, that is known to activate HIV-1 replication in several model systems, can play a major role in the observed inhibition of HIV-1 replication.

Key words: Ridostin; C8166 cells; HIV-1 replication; cytokines

Introduction

The biological response to inflammatory agents is regulated by a complex network of stimulatory and inhibitory cytokines production by various cell types, including monocytes, T and B cells, fibroblasts and endothelial cells (Arai *et al.,* 1990). HIV-1 infection induces a profound dysfunction of the immune response, leading to abnormal production of several cytokines (Pandolfi, 1993). The only agents found to be clinically useful in the treatment of acquired immunodeficiency syndrome (AIDS) patients to date are reverse transcriptase (RT) inhibitors (Connolly and Hammer, 1992), that include the nucleoside analogs (Furman *et al.,* 1986) as well as a class of structurally diverse non-nucleoside RT inhibitors (Buckheit *et al.,* 1994). However, the therapy with these inhibitors, including azidothymidine (AZT), is limited by adverse reactions and development of resistant strains during treatment (Richman *et al.,* 1987; Larder *et al.,* 1989). Furthermore, the HIV infection is suppressed but not cured, and HIV disease, although delayed, invariably progresses (O'Marro *et al.,* 1992). Other types of therapeutical agents able to strengthen the immune system are being investigated.

It has been recently pointed out that an application of exogenous dsRNA to patients with AIDS-related complex (ARC) and AIDS may help to restore the natural dsRNA-dependent pathway (2',5'-oligo-A-synthetase/RNAse L system and dsRNA-dependent protein kinase) and to overcome the depressed immune status of the patients. Such regulatory mechanism may interfere with HIV replication and the antiviral effect would greatly depend on the capacity of the cells to active this pathway (Gochi *et al.,* 1992). Furthermore, it has been recently shown that the antiviral effect of dsRNA may be modulated by viral proteins, e.g. the HIV-1 Tat protein (Schroder *et al.,* 1990).

Abbreviations: AIDS = acquired immunodeficiency syndrome; ARC = AIDS-related complex; AZT = azidothymidine; HIV = human immunodeficiency virus; IFN = interferon; MoAb = monoclonal antibody; MOI = multiplicity of infection; TNF = tumor necrosis factor; RT = reverse transcriptase

PolyI:polyC is the best known nucleotide inducer of IFN, and it is known to activate *per se* the 2',5'-oligo-A-synthetase (Ilson *et al.*, 1986). However, polyI:polyC is toxic (Krown *et al.*, 1983). This disadvantage has ben overcome by the development of mismatched dsRNA with low cytotoxicity (Carter *et al.*, 1987; Montefiori *et al.*, 1987). Alternatively, natural dsRNA, such as Ridostin (dsRNA preparation from *S. cervisiae*), has been shown to exert some anti-HIV effects without strong toxicity (Nossic *et al.*, 1992a). Here we show that Ridostin is able to inhibit HIV-1 replication in a T lymphoblastoid cell line C8166. A concomitant alteration of the levels of cytokines spontaneously released by this cell line was observed upon Ridostin treatment.

Materials and Methods

Ridostin was a gift of Dr. V. Masycheva, The Biologically Active Substances Research and Technology Institute, Berdsk, Russia. This preparation was isolated from lysates of killer yeast *S. cerevisiae*. It is a powder soluble in water and physiological solution, and it contains 40% of nucleotides, 12% of dsRNA, 48% of NaCl, and is free of DNA, proteins and carbohydrates. Ridostin is an inducer of IFN in animals and in cell cultures *in vitro* and exerts immunomodulating properties in mouse and human models (Ershov *et al.*, 1993). MoAb to TNF alpha was kindly provided by Dr. G. Adolf, Bender and Co. Wien, Austria.

Cells and virus. Human T lymphoblastoid cell line C8166 was grown as a suspension culture in RPMI-1640 medium (Clontech Hy Clon, Palo Alto, CA) supplemented with 10% foetal calf serum (FCS). Cultures were maintained at 37 °C in humidified atmosphere of 5% CO_2. Cells were routinely seeded at a concentration 2.5 x 10^5/ml, infected with HIV-1 strain P1 in a small volume at a multiplicity of infection (MOI) dependent on the aim of the experiment. After 1 hr incubation at 37 °C the cells were washed 3 times, resuspended at 5 x 10^5/ml and incubated at 37 °C. Cultures were sampled at different time points to measure virus replication. Doses and time for Ridostin addition were chosen as detailed in the description of the various experiments. HIV-1 strain P1 was a strongly cytopathogenic virus strain obtained from a clinical isolate. It was grown in C8166 cells and extracted from the infected cultures by cell cryolysis, as described by Dianzani *et al.* (1988).

Titration of HIV-1 infectivity. Cell suspensions were lysed by 3 cycles of cryolysis. Infectious virus present in the supernatant of lysates following centrifugation was titrated as described by Dianzani *et al.* (1989). Briefly, 100 µl aliquots of serial dilutions of supernatant samples were mixed with 7 x 10^4 C8166 cells in 100 µl of the growth medium in wells of microtiter plates. After 4 days of incubation at 37 °C the cultures were scored for CPE, appearing as large syncytia.

ELISA of cytokines. To determine the amount of cytokines present in the supernatant of C8166 cells either treated with Ridostin or untreated, commercial ELISA kits were used (Ameglio *et al.*, 1994).

MTT test was used to check the toxicity of Ridostin in C8166 cells by the method of Denizot *et al.* (1986). Briefly, to each well of microtiter plates 2-fold dilutions of Ridostin and 5 x 10^4 of C8166 cells were added in 200 µl of the growth medium. After 2 days of incubation, 100 µl of medium was discarded and 20 µl of MTT reagent 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide was added to each well. After 2 hrs of incubation in the dark at 37 °C, 150 µl of isopropanol was added and A_{570} and A_{700} were recorded.

Results

C8166 cells were infected with HIV-1 at three different MOI and treated with Ridostin. The results of one of these experiments (Fig. 1) show that Ridostin dose-dependently inhibited HIV-1 replication, the 50% inhibitory concentrations (IC_{50}) of Ridostin being 4.5, 11 and 68 µg/ml at MOI 0.01, 0.001, 0.0001 $TCID_{50}$/cell, respectively. Ridostin at these concentrations was not toxic for C8166 cells as indicated by MTT test, showing that Ridostin caused only a 30% growth reduction at a concentration of 4 mg/ml (Fig. 2). According to these results the selectivity index of Ridostin was above 100.

As it has been shown that Ridostin induces IFN alpha in some cell cultures (Nossic *et al.*, 1992b), in the subsequent experiments C8166 cells were treated with Ridostin and the production of some cytokines (namely IFN alpha and gamma, and TNF alpha) was assayed. It can be seen from Table 1 that C8166 cells spontaneously released low levels of IFN alpha and gamma, as well as TNF alpha. Ridostin induced an enhancement of IFN alpha production from 2.1 to 18.3 U/ml on the first day, and from 4.3 to 31.7 U/ml on the second day of induction. The effect on IFN gamma and TNF alpha release was opposite. In fact, the level of IFN gamma decreased from 2.15 to 0.2 U/ml on the first day, and from 8.49 to 0.74 U/ml on the second day. Similarly, the amount of TNF alpha decreased from 63 to 39 pg/ml on the first day, and from 548 to 85 pg/ml on the second day in cultures trated with Ridostin.

Table 1. Effect of Ridostin on cytokines release by C8166 cells

Treatment	IFN alpha	TNF alpha	IFN gamma
None (day 1)	2.1 ± 0.6	73.0 ± 8.0	2.1 ± 1.2
Ridostin (day 1)	18.3 ± 4.2	39.0 ± 5.0	0.2 ± 0.1
None (day 2)	4.3 ± 1.2	548.0 ± 40.0	8.49 ± 2.3
Ridostin (day 2)	31.7 ± 6.2	85.0 ± 10.0	0.74 ± 0.2

Cells (5 x 10^5/ml) were incubated with Ridostin (400 mg/ml). Supernatants were collected at days 1 and 2 and assayed for IFN alpha 2, IFN gamma and TNF alpha.

TNF alpha has been shown to active HIV-1 replication in different cell systems (Okamoto *et al.*, 1989; Osborn *et al.*, 1989). Therefore we hypothesized that a reduction of TNF alpha could be involved in the effect of Ridostin in this experimental system. To establish whether a reduced amount of TNF alpha could initiate an inhibition of HIV-1 replica-

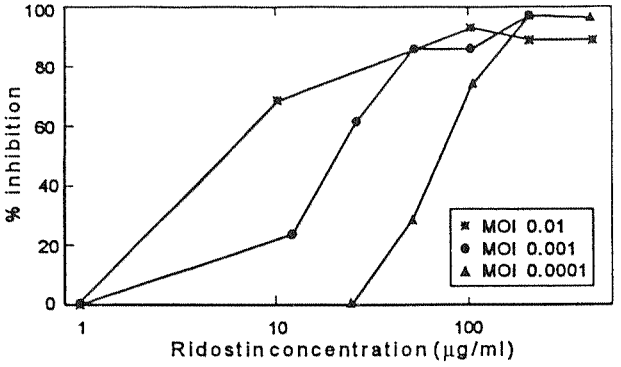

Fig. 1
Effect of Ridostin on replication of HIV-1 in C8166 cells
5 x 10^5 cells in 50 μl of medium were infected with HIV-1 at different MOI. After 1 hr incubation at 37 °C, the cells were extensively washed and resuspended at 5 x 10^5 cells per ml. 500 μl aliquots of cell suspensions were seeded into wells of 24-well plate and Ridostin in different concentrations in 25 μl of medium was added. Three days later, when control cultures showed large syncytia, cell suspensions were frozen and thawed 3 times and titrated for infectious virus.

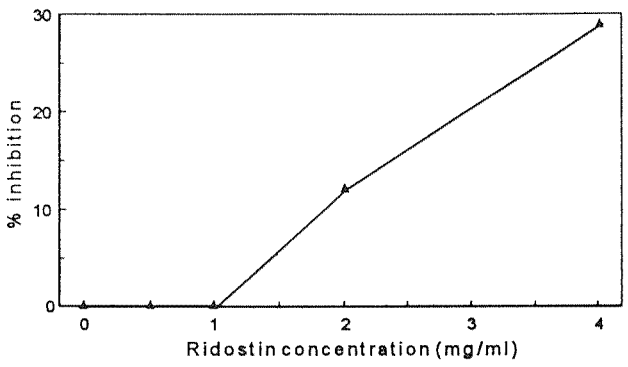

Fig. 2
Inhibition of growth of C8166 cells by Ridostin
C8166 cells were incubated at 37 °C with Ridostin in different concentrations. The cell viability was assayed 2 days later by the MTT staining.

tion, we measured the HIV-1 replication in C8166 cultures exposed to anti-TNF alpha MoAb which could neutralize TNF alpha endogenously produced by these cells.

The results (Table 2) indicate that the neutralization of TNF alpha resulted in a dose-dependent inhibition of HIV-1 yield by C8166 cells, although the inhibition was observed only at very high concentration of the MoAb.

Table 2. Effect of MoAb to IFN alpha on HIV-1 replication in C8166 cells

MoAb to TNF alpha (μg/ml)	HIV-1 yield (log $TCID_{50}$/ml)
0	3.25
1	1.75
5	1.12
10	<0.5

Cells were infected with HIV-1 at MOI of 0.001 $TCID_{50}$/cell in 50 μl of medium. After 1 hr at 37 °C the cells were extensively washed, resuspended in medium at 5 x 10^5/ml and different amounts of MoAb to TNF alpha were added. Infectious virus in cryolysates of cultures was assayed 3 days later.

Discussion

In the present paper the effects of Ridostin on HIV-1 replication in acutely infected cells was studied. Ridostin reduced HIV replication in the T lymphoblastoid cell line C8166 in a dose-dependent manner. The ID_{50} was dependent on the MOI and ranged between 4.5 and 68 μg/ml. The fact that ID_{50} and MOI values were inversely proportional seems to be surprising; nevertheless, we may assume that an earlier appearance of syncytia at higher MOI resulted in a poorer virus replication. Since the IC_{50} of Ridostin for C8166 cells growth was above 4 mg/ml, the selectivity index was above 100. These data suggest that Ridostin is suitable for further investigation as a possible anti-HIV-1 preparation.

Ridostin has been shown to induce IFN alpha production (Nossic *et al.*, 1992a). In C8166 cells, that spontaneously producedTNF alpha, IFN alpha and small amounts of IFN gamma, Ridostin treatment resulted in the increased IFN alpha release and the decreased TNF alpha and IFN gamma production. It has been shown that IFN-induced RNA-binding proteins such as 2',5'-oligo-A-synthetase, pI/eIF2 kinase and endonuclease L play a role in IFN-mediated inhibition of viral infections (Samuel, 1991). However, the induction of these RNA-binding proteins does not appear to play a role in limiting the replication of HIV-1 in several systems. E.g., in T cells and macrophages chronically infected with HIV-1, IFNs appeared to act at the post-translational level by interfering with virus particle assembly and release (Poli *et al.*, 1989). In the continuous cell line HeLa-CD4, the post-transcriptional inhibition of HIV-1 replication by IFN alpha 2 was highly ineffective and could be observed only in the absence of the Tat protein (Shirazi *et al.*, 1994). For these reasons we suppose that the effects of Ridostin could not be mediated by the induction of IFN alpha, to which C8166 cells are resistant (O. Scheglovitova, unpublished results), but by the alteration of the levels of other cytokines that are known to affect HIV-1 replication (e.g. TNF alpha). In fact, we observed that the level of TNF alpha was reduced in C8166 cell cultures exposed to Ridostin. In our experimental system, MoAb to TNF alpha exerted a dose-dependent inhibition of HIV-1 replication in C8166 cells. Therefore we think that the Ridostin-mediated inhibition of HIV-1 replication in C8166 cells can be attributable, at least in part, to reduced levels of endogenous TNF alpha.

In conclusion, our data indicate that Ridostin can be envisaged as a preparation that inhibits HIV-1 replication *in vitro*, possibly by altering cytokine production, and can be recommended for future investigation aimed to increase the number of compounds that could be effective in controlling HIV replication *in vivo*.

Acknowledgements. This study was performed during a research stay of one of the authors (O.S.) at the Institute of Virology, University of Rome "La Sapienza", Rome, Italy, as a fellow supported by Italian Health Ministry grant to F.D. AIDS Project No. 930453. We gratefully acknowledge helpful suggestions and support by Dr. M.R. Capobianchi and Prof. F. Dianzani, Institute of Virology, Rome, Italy.

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