Antiviral activity of a chemically stabilized 2–5A analog upon microinjection into HeLa cells

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2–5A[ppp(A2′p)$_n$5′A] has been implicated as a mediator in the antiviral action of interferon. Its direct evaluation as an indicator of virus replication is hampered by two limitations: its inability to penetrate intact cells, and its rapid intracellular degradation by (2′–5′)phosphodiesterase. These problems could be overcome by using a microinjection technique whereby a phosphodiesterase-resistant analog of 2–A, in which the 2′-terminals adenosine residue is replaced by 2-(9-adenyl)-6-hydroxy-methyl-4-hexylmorpholine, was injected into individual HeLa cells before infection with mengovirus or vesicular stomatitis virus (VSV). This comparative assay with two representatives of different virus classes in a single experimental system pointed to the high sensitivity of VSV to inhibition by 2–5A oligonucleotides, in contrast with the low sensitivity of mengovirus. Microinjection of the hexylmorpholine 2–5A analog led to a much greater reduction in mengovirus yield than did microinjection of 2–5A itself.

Antiviral ativity *Microinjection* *2–5A analog* *Interferon*

1. INTRODUCTION

In the presence of dsRNA, 2–5A synthetase(s) convert ATP to a series of unusual oligonucleotides with the general structure ppp(A2′p)$_n$5′A (n = 1–14), referred to as 2–5A [1–4]. These oligonucleotides specifically activate an endoribonuclease, RNase L, that is responsible for the cleavage of mRNA [5] and rRNA [6]. Some structural requirements for the activation of this nuclease in vitro have been established [7–18]. The production of 2–5A has been demonstrated in several interferon (IFN)-treated virus-infected cell systems; in IFN-treated EMC virus-infected L cells, both the triphosphate (2–5A) and its core were detected by HPLC, radioimmuno and radiobinding assays, and the appearance of these products correlated with the establishment of the antiviral state [7–19]. That RNase L is activated under these conditions was attested to by the degradation of rRNA and EMC virus RNA [20,21]. Furthermore, the synthesis of 2–5A seems to protect IFN-treated HeLa cells infected with reovirus and is accompanied by reovirus mRNA

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Abbreviations: dsRNA, double-stranded RNA; 2–5A, 2′–5′-oligonucleotides with the general structure ppp(A2′p)$_n$5′A (n = 2 → trimer, n = 3 → tetramer); core 2–5A, 5′-dephosphorylated 2–5A, thus (A2′p)$_n$5′A; RNase L, 2–5A-activated endoribonuclease, also called ribonuclease F; EMC virus, encephalomyocarditis virus; xylo 2–5A core, xyloadenosine analogue with the general structure (xyloA2′p)$_2$xyloA; hexylmorpholine 2–5A analog, 2–5A in which the ribose of the 2′-terminal nucleotide is transformed to an *N*-hexylmorpholine (azahexapyranose), specifically, 2-(adenyl)-6-hydroxymethyl-4-hexylmorpholine

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degradation [22]. A similar situation may occur in vaccinia virus-infected cells [23] and Semliki Forest virus-infected cells [24]. However, in a number of virus-cell systems, the contribution of the 2–5A-mediated activation of RNase L to the overall antiviral activity could not be ascertained ([25–27]; reviews [28,29]).

Attempts have been directed at stimulating the uptake of 2–5A oligonucleotides in intact cells by permeabilising the cells, either by hypertonic salt treatment or calcium phosphate precipitation, or by incubating the cells with high concentrations of 2–5A core [6–8]. However, such procedures did not permit quantitative assessment of the structural requirements of the 2–5A analogs for RNase L activation, i.e. because of the membrane damage ensuing from these procedures as well as the low stability of 2–5A itself [8,30–32].

At least two other enzymes are involved in the 2–5A pathway: (i) a phosphodiesterase, which specifically cleaves (2′–5′)-phosphodiester linkages, starting at the 2′-terminus; the induction of this enzyme by IFN has been demonstrated only in mouse L cells [33] and its role in intact cells is not yet understood [34]; (ii) one or several phosphatases able to dephosphorylate 2–5A to its core; the occurrence of this (these) enzyme(s) in intact cells are/is also not clear [28]. The presence of these two enzymes may account for the rapid degradation of 2–5A in intact cells and cell extracts. Therefore, several analogs of 2–5A have been synthesized either chemically or enzymatically with the aim to increase their metabolic stability toward degradative enzymes [11,12,14,15,35].

In fact, the 2′-terminal ribose and in particular its 3′-OH group are not required for either RNase L binding or activation [9], although the free 2′- and 3′-OH groups of the 2′-terminal ribose are essential for degradation by phosphodiesterase. Imai et al. [36] have transformed the 2′-terminal ribose of 2–5A to an *N*-substituted morpholine (azahexapyranose) [The analog used in these studies was a tetraadenylate in which the 2′-terminal adenosine residue was converted to 2-(9-adenyl)-6-hydroxymethyl-4-hexylmorpholine, also referred to as 9-(3′-aza-4′-hexyl-1′,2′,3′,4′-tetradeoxyhexopyranos-1′-yl)adenine. For the sake of convenience it will be referred to as 'tailed' 2–5A analog.], thus obtaining a molecule resistant to phosphodiesterase degradation without affecting RNase L binding or activation. As a triphosphate, this 2–5A analog cannot be taken up by the cells unless they are permeabilized, and so far the activity of this analog has been investigated only in cell-free systems [36].

Here, we have applied the microinjection technique [37,38] to investigate the inhibitory effects of the tailed 2–5A analog on the replication of mengovirus and vesicular stomatitis virus (VSV) in HeLa cells.

2. EXPERIMENTAL

2.1. *2′–5′ Oligoadenylates*

The oligonucleotide trimer and tetramer 5′-triphosphate ppp(A2′p)$_2$A and ppp(A2′p)$_3$A were obtained from PL-Biochemicals.

The *N*-substituted morpholine analogs or tailed analogs (tetramer 5′-triphosphate and monophosphate) have been described previously [36].

2.2. *Tailed tetramer core*

The tailed tetramer core was prepared as follows: the triethylammonium salt of tailed tetramer monophosphate (220 A_{260} units, 5.3 μmol), prepared according to Imai et al. [36], was digested with bacterial alkaline phosphatases (0.5 units) in 500 μl of 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$ at 37°C for 2.5 h. At the termination of the incubation, the reaction mixture was heated to 100°C for 5 min and then centrifuged at 10000 × *g*. The supernatant was then applied to a DEAE-Sephadex A25 column (HCO_3^- form, 1 × 20 cm) which was subsequently eluted with a linear gradient of 0–0.15 M triethylammonium bicarbonate (pH 7.6, total volume 500 ml). Appropriate fractions were pooled and evaporated to dryness in vacuo. The residue was taken up in water and the resulting solution was evaporated. This process was repeated several times to remove all triethylammonium bicarbonate. This residue was redissolved in dry methanol (200 μl), and the solution added dropwise to a stirred solution of NaI in acetone (0.1 M, total volume 2.0 ml). The precipitate was isolated by centrifugation, washed (3 ×) with dry acetone and then dried over P_2O_5 for 2 h. The yield of tailed tetramer core was 202 A_{260} units (4.9 μmol, 92%).

2.3. *Viruses and cells*

VSV was grown in human amniotic cells (UAC) and mengovirus was grown in L929 cells, maintained in minimum essential medium (MEM) supplemented with 2% fetal calf serum (FCS) and stored in aliquots at −70°C. Virus titers were 2.5×10^8 PFU/ml (VSV) and 2.3×10^8 PFU/ml (mengo).

HeLa cells were grown in RPMI 1640 medium supplemented with 10% FCS. For the microinjection procedure, HeLa cells were cultivated on small glass plates (2 mm^2) dispersed in 6 cm petri dishes in MEM plus 10% FCS.

For the plaque assay L929 cells were cultured in MEM supplemented with 10% FCS and then dispensed in aliquots of 1.2×10^6 cells in the same medium into 60 mm diameter Falcon dishes.

The two cell lines were incubated at 37°C in a 5% CO_2 humidified atmosphere.

2.4. *Cell microinjection*

Microinjection of the compounds into HeLa cells was performed according to Graessmann and Graessmann [37], using glass capillaries (tip diameter about 0.5–1 μm) [31,32]. In each experiment approx. 100–400 cells were microinjected with 5×10^{-10} ml/cell. Injections were performed under a phase contrast microscope [37,38].

2.5. *Infection*

Each glass plate of microinjected cells was transferred onto a microwell plate and infected with 200 μl of virus dilution, so as to obtain a multiplicity of infection (MOI) of 10, just after microinjection or at the time indicated in the experiment (in the latter case the cells were maintained in RPMI with 10% FCS until infection). After 1 h incubation at 37°C, the virus was removed by washing 3 times with the same medium and then each well was replenished with 200 μl of RPMI + 10% FCS. The cells were enumerated and incubation at 37°C continued for another 20 h. At this time, the supernatant was collected and stored at −70°C.

2.6. *Antiviral activity*

The antiviral activity was determined by a monolayer plaque assay: L929 cell monolayers were incubated for 1 h with 200 μl of each sample diluted 10–10^5-times. After 1 h adsorption, 5 ml melted agar medium (Indubiose IBF 1.8%-MEM 2 × 1:1 + 2.5 FCS) was added to each 60 mm well. The number of plaques was counted and the titer was determined after 1–2 days incubation and staining with neutral red (0.2% in PBS).

Table 1

Dose-response relationship of the antiviral activity of the 2–5A hexylmorpholine triphosphate analog against VSV

Concentration (μM)	Number of cells	PFU/ml	PFU/cells	% of control[a]
Expt 1				
100	160	4×10^3	25	1.25
10	141	1.2×10^4	85.1	4.2
1	190	8×10^3	42.1	2.1
0[a]	200	4×10^5	2000	100
Expt 2				
1	135	2×10^2	1.48	0.13
0.1	144	8×10^2	5.5	0.5
0.01	110	3×10^3	27.2	2.5
0[a]	82	9×10^4	1097.5	100

[a] For each experiment the % of control was calculated as a function of the value of PFU/cells obtained for VSV-infected HeLa cells which had not been microinjected

3. RESULTS AND DISCUSSION

By using microinjection of 2–5A oligonucleotides in intact cells, before virus infection, it should be possible to assess directly to what extent these oligonucleotides are able to suppress virus replication. This technique circumvents the problem that 2–5A molecules by virtue of their 5′-triphosphate group cannot penetrate the cells from the outside. Furthermore, the microinjection technique permits a study of the interplay of degradative enzymes, i.e. (2′–5′)-phosphodiesterase, with the in situ antiviral effects of the 2–5A oligonucleotides.

The microinjection of 2–5A hexylmorpholine analogs into HeLa cells before infection with VSV or mengovirus at an MOI of 10 has enabled us to compare directly the inhibitory effects of 2–5A oligonucleotides on different classes of viruses. At the concentration used neither the natural 2–5A trimer or tetramer triphosphates nor their hexylmorpholine-tailed analogs impaired cell viability. Microinjected cells were maintained in culture for 3 days without any detectable change in their rate of division. In a dose-response experiment (table 1) a complete inhibition of VSV growth was found when the 2–5A hexylmorpholine analog triphosphate was microinjected at 10 nM (representing 1 nM inside the cells, considering the total volume injected and the average volume of the cell). This is at least 20-fold lower than the concentration of natural 2–5A in IFN-treated EMC virus-infected L cells [6]. These results confirm previous observations that VSV growth is sensitive to inhibition by natural 2–5A [39,40] and 2′-*O*-phosphoglyceryl derivatives of 2–5A [41], although the 2–5A system does not appear to be activated in intact cells [42,43].

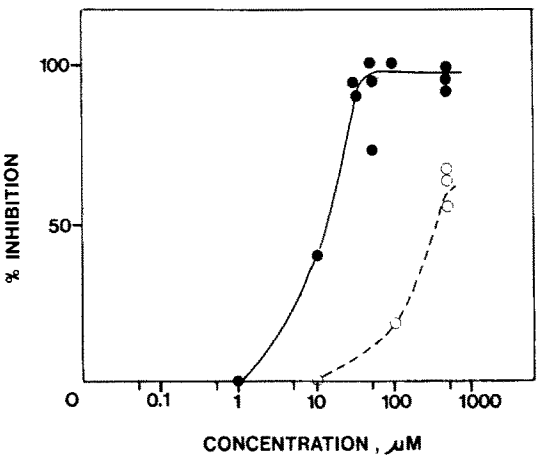

Fig.1. Dose-response relationship of the antiviral activity of natural 2–5A and its tailed analog, both microinjected immediately before mengovirus infection. Concentrations of (2′–5′)pppA$_4$ (O---O) and tailed (2′–5′)pppA$_4$ (●——●) are indicated on the abscissa.

The present data confirm the ability of 2–5A to block VSV replication, when the oligonucleotide is present at the beginning of the infection [40]. Upon microinjection of natural 2–5A complete inhibition of VSV growth was achieved at 100 nM–1 μM 2–5A inside the cell (not shown) [39,41]. The work presented here shows that in intact cells the tailed analogs are at least 100-fold more active than natural 2–5A. Moreover, the activity of the 2–5A hexylmorpholine analog towards VSV is at least 1000-fold higher than that obtained with the phosphoglyceryl derivatives of 2–5A [41], although the two analogs have comparable activity and stability in an in vitro assay [36,41].

Since 2–5A has not been detected in IFN-treated VSV-infected cells [42,43], it was of interest to investigate the antiviral activity of 2–5A (or its analogs) towards other types of viruses, known to activate the 2–5A pathway in the presence of IFN in intact cells [6,19]. Thus, we tested 2–5A and its hexylmorpholine analog on mengovirus, a picornavirus, belonging to the same group as the extensively studied EMC virus. Paradoxically, microinjection of natural 2–5A and even its hexylmorpholine analog proved poorly active in inhibiting mengovirus growth.

When 500 μM solutions of the natural 2–5A trimer or tetramer triphosphates were microinjected – representing a concentration of about 50 μM inside the cell – (2′–5′)pppA$_3$ and (2′–5′)pppA$_4$ achieved about 60% inhibition in the production of infectious mengovirus particles. Furthermore, microinjection of HeLa cells with a 500 μM solution of the tailed 2–5A tetramer triphosphate analog resulted in an almost complete inhibition of mengovirus replication (fig.1).

The inhibitory effects of natural 2–5A and its tailed analog on mengovirus replication were then directly compared in a dose-response experiment. Fig.1 shows that microinjection of a 100 μM solution of tetramer 2–5A triphosphate gave an inhibi-

Table 2

Antiviral activity against VSV and mengovirus of the 2–5A hexylmorpholine core and monophosphate analogs

	Concentration (μM)	Number of cells	PFU/ml	PFU/cells	% of control[a]
Expt 3: Mengovirus					
Core	500	60	2.5×10^4	416.6	8.8
	500	52	5×10^4	961.5	20.4
Monophosphate	500	239	6.9×10^4	288.7	6.1
	0[a]	180	8.5×10^5	4722	100
Expt 4: VSV					
Core	100	124	1×10^4	80.6	520
	10	155	2×10^4	129	832.3
	1	112	4×10^3	35.7	230.4
	0[a]	129	2×10^3	15.5	100

[a] For each experiment the % of control was calculated as a function of the value of PFU/cells obtained for virus-infected HeLa cells which had not been microinjected

tion of only 20% and no inhibition was found at 10 μM. For the tailed analog, maximum inhibition of virus replication was achieved at 30 μM (3 μM intracellular) and above; 50% inhibition was obtained upon microinjection of a 15 μM solution (about 1.5 μM intracellular). Since maximal inhibition of mengovirus replication by tailed 2–5A was achieved only at a rather high concentration (30 μM intracellularly) (fig.1), i.e. at least 150-fold higher than that of natural 2–5A in IFN-treated EMC virus-infected L cells [6], we conclude that mengovirus was relatively refractory to 2–5A and its analogs. This high resistance of mengovirus to the antiviral effect of 2–5A is in keeping with previous observations showing the lack of 2–5A-dependent RNase activation by 2–5A in cell-free extracts obtained from EMC-infected cells [44,45]. The present results suggest that this situation may also occur in intact cells. However, other hypotheses could be considered to explain the lower sensitivity of mengovirus to the antiviral effect of 2–5A, such as a more difficult diffusion or access of the oligonucleotides to the viral replication site in mengovirus-infected cells [46,47].

Part of the improvement of antiviral activity towards mengovirus observed here with microinjected hexylmorpholine analog could be due to catabolic products liberated by the intracellular processing of the tailed analog. This is confirmed by the microinjection of the core and the monophosphate hexylmorpholine analogs, which gave maximal inhibition of mengovirus growth at 500 μM (table 2). In contrast, VSV (shown in our experimental system to be an indicator of the 2–5A-dependent RNase activation) was completely resistant to different concentrations of the tailed-core analog (table 2). Since it is known that core 2–5A neither binds [19] nor activates [29] the 2–5A-dependent RNase, this also indicates that the inhibition of mengovirus replication by the latter analogs might be unrelated to the activation of the 2–5A-dependent endonuclease.

The microinjection technique represents a powerful tool to monitor in situ the antiviral effects of 2–5A analogs as well as their interplay with different enzymes, be it anabolic or catabolic. By this technique any new 2–5A analog [48,49] can be monitored for its fate within the cell. The microinjection technique may thus have an important predictive advantage in terms of quantitative biological activity of various 2–5A analogs in intact cells.

Finally, the microinjection technique offers the possibility of investigating the effects of 2–5A

derivatives on different classes of viruses independently of the physiological role they could play in the 'natural' response to interferon.

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