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ANTIVIRAL AND ANTICELLULAR EFFECTS OF SYNTHETIC (2'-5')-OLIGOADENYLATE (A 2' p 5' A 2' p 5' A) IN RAUSCHER MURINE LEUKAEMIA

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Summary. — Antiviral and antileukaemic effects of the synthetic (2'-5')-oligoadenylate trimer [(2'-5')-ApApA] were demonstrated in BALB/c mice infected with Rauscher murine leukaemia virus (RMLV) by intraperitoneal (i.p.) treatment for 5-20 days (100 μg — — 1 mg daily doses) as evidenced by 72% suppression of viraemia and by decreased activity of serum reverse transcriptase levels. Electron microscopy revealed more than 95% inhibition of RMLV replication as compared to controls in transformed spleen cells from mice treated 5 times with 1 mg dose of (2'-5') ApApA. A significant and dose-dependent reduction of spleen weights of the RMLV-infected mice treated with (2'-5') ApApA was also observed. The antileukaemic effect of (2'-5-') ApApA was enhanced by simultaneous i.p. injection of amphotericin B (20 μg/mouse). In comparison to the effect of interferon (IFN) on RNA tumour viruses, our results suggest a higher antiviral activity of the synthetic (2'-5') ApApA oligonucleotide in suppressing RMLV replication in vivo.

Key words: (2'-5')-oligoadenylate trimer; inhibition; Rauscher murine leukaemia virus replication; BALB/c mice; electron microscopy; reverse transcriptase

Introduction

Treatment of cells with IFN induces at least two enzymatic activities, (2'-5')-oligoadenylate synthetase, and dsRNA-dependent protein kinase (Lebleu *et al.*, 1976; Roberts *et al.*, 1976; Hovanessian *et al.*, 1977; Zilberstein *et al.*, 1978; reviewed by Baglioni, 1979). (2'-5')-oligoadenylate synthetase catalyzes the polymerization of ATP into a series of Oligoadenylic acid molecules with the general structure pppA(2' p 5' A)$_n$ (Kerr and Brown, 1978; Martin *et al.*, 1979). These oligonucleotides activate a ribonuclease (RNase F) in vitro which is present in the cell extract as an inactive enzyme (Slatery *et al.*, 1979; Nilsen *et al.*, 1981). The active RNase F has been correlated

with both the antiviral and the anticellular effects of IFN, since it was shown to degrade both viral and cellular RNA in vitro (Schmidt *et al.*, 1978; Nilsen and Baglioni, 1979; Kimchi *et al.*, 1981).

Several investigators reported that (2'-5')-oligoadenylates (trimer or higher oligomers) added exogenously to cell cultures inhibited the synthesis of cellular proteins, RNA and DNA (Williams and Kerr, 1978; Hovanessian *et al.*, 1979; Hovanessian and Wood, 1980; Baglioni *et al.*, 1981; Jurovčík and Smrt, 1981). In addition, vesicular stomatitis virus replication was suppressed in the pppA(2' p 5' A)$_n$-treated and VSV-infected cultures and (Hovanessian and Wood, 1980). Thus, the pppA (2' p 5'A)$_n$ was proposed to serve as intermediate molecule for both the antiviral and anticellular activities of IFN.

Our group has recently reported the inhibitory effect of exogenous synthetic (2'-5')-oligoadenylate, A 2' p 5' A 2' p 5' A, (2—5 A core), on the protein synthesis in intact mouse lymphocytes, hepatocytes, and bone marrow cells (Jurovčík and Smrt, 1981). Antimitogenic effect of similar synthetic (2'-5')--oligoadenylates dephosphorylated at the 5'-end has been shown in synchronized 3T3 fibroblasts (Kimchi *et al.*, 1981*a*). The present paper brings experimental evidence on the antiviral and anticellular activity of synthetic (2'-5')-oligoadenylate trimer, (2'-5')ApApA, administered to BALB/c mice infected with RMLV (Rauscher, 1962). Our results demonstrate a significant inhibition of intracellular RMLV replication in vivo by exogenous (2'-5')ApApA, contrasting with the post-translational model of IFN action on type-C retroviruses, in which interference with virus assembly, maturation and infectivity was not accompanied by decreased number of intracellular virions (Friedman and Ramseur, 1974; Billiau, 1977; Billiau *et al.*, 1978; Pitha *et al.*, 1979, 1980).

Materials and Methods

The (2'-5')-oligoadenylate trimer, (2'-5')ApApA was prepared as described (Karpeisky *et al.*, 1982).

Rauscher erythroleukaemia was induced in BALB/c male mice by i. p. injection of 0.2 ml of RMLV preparation (Kára *et al.*, 1974). Progression of the disease was tested by the rate of splenomegaly, the spleen weight of infected animals being determined 21 days post infection (p.i.). The infected animals were inoculated i.p. with (2'-5')ApApA in 0.2 ml of sterile isotonic saline in concentrations and time schedule described in Table 1. Amphotericin B (Fungizone, Squibb), 20 μq in 0.2 ml of isotonic saline per mouse, was used in some experiments in order to increase the permeability of cell membranes (Kotler-Brajtburg *et al.*, 1977).

Reverse transcriptase (RT) activity as a marker of viraemia in sera of the RMLV-infected mice treated with (2'-5')ApApA and in controls was determined as described earlier (Kára *et al.*, 1972). The sera of animals in each group were pooled (21 days p.i.), and virus present in 1 ml of pooled serum was pelleted by centrifugation at 100,000 × g for 1 hr (4 °C). The viral pellet was resuspended in 0.1 ml of 0.1 mol/l Tris-HCl buffer (pH 8.2), containing 80 mmol/l KCl, 10 mmol/l $MgCl_2$, 5 mmol/l dithiothreitol and 0.1% Triton X-100. The virions were lysed in this buffer at 0 °C for 1 hr and the reverse transcriptase activity in 50 μl of the lysate was determined using 50 μg poly(rA).oligo (dT)$_{10}$ (Calbiochem) and 185 kBq thymidine-(methyl-^{3}H)-5'-triphosphate (sp. act. 976 GBq/mmol, Amersham) per assay (Kára *et al.*, 1972). RT activity was expressed as c.p.m. of thymidine-(methyl-^{3}H)-5'-triphosphate incorporated into the DNA strand by viral RNA-directed DNA synthesis after 1 hr incubation at 37 °C.

Table 1. Inhibition of splenomegaly and viraemia by (2'-5') ApApA in BALB/c mice infected with Rauscher murine leukaemia virus (RMLV)

Animal group	Treatment	Number of doses (days p.i.)	No. of animals per group	Average spleen weight (mg)	Inhibition of splenomegaly (%)	RT-activity in serum (% of control)
	Experiment I					
1	Controls (0.2 ml saline)	5 (1—5)	10	849	—	—
2	(2'—5')ApApA (1 mg/mouse)	5 (1—5)	10	697	17	—
3	(2'—5')ApApA (1 mg/mouse) + amphotericin B (20 μg/mouse)	5 (1—5)	10	627 ($P < 0.05$)	26	—
4	(2'—5')ApApA (0.5 mg/mouse) + amphotericin B (20 μg/mouse)	5 (1—5)	10	658 ($P < 0.1$)	23	—
	Experiment II					
1	Controls (0.2 ml saline)	15 (1—19)	10	851	—	—
2	(2'—5')ApApA (2 mg/mouse)	15 (1—19)	10	686	20	—
3	(2'—5')ApApA (1 mg/mouse)	15 (1—19)	10	706	17	—
	Experiment III					
1	Controls (0.2 ml saline)	15 (1—19)	10	761	—	100
2	(2'—5')ApApA (100 μg/mouse) + amphotericin B (20 μg/mouse)	15 (1—19)	10	633	17	28
3	Amphotericin B (20 μg/mouse)	15 (1—19)	10	714	6	165

The average spleen weitht in the groups of animals was determined on day 21 p.i.
In the experiments II and III, i.p. injections were given on days 1—5, 8—12 and 15—19 p.i.

Electron microscopic analysis of spleen cells from RMLV-infected animals in the (2'-5')ApApA--treated and control groups was performed as described (Mach and Libánský, 1971). Briefly, the tissue samples were fixed in OsO_4, postfixed with glutaraldehyde and, after dehydration, embedded into Araldite. Ultrathin sections from several parts of spleen tissue were stained with lead citrate and uranylacetate and observed in a JEM 7 electron microscope.

Results

The antileukaemic effect of different doses of (2'-5')ApApA given i. p. in a different time schedule to the RMLV-infected BALB/c mice is demonstrated in Table 1. Five i. p. injections of (2'-5')ApApA (40 mg/kg, i.e. 1 mg per mouse daily on five consecutive days) reduced the spleen weights significantly, the average spleen weight in this group being 17% lower in comparison with controls. Prolonged treatment with a higher dose (80 mg/kg, i. e., 2 mg per mouse daily on 15 consecutive days) increased only slightly the inhibitory effect of (2'-5')ApApA on splenomegaly.

Amphotericin B (20 μg per mouse) administered i. p. simultaneously with (2'-5')ApApA enhanced significantly its antileukaemic effect. In the group treated with 5 doses of (2'-5')ApApA (1 mg per mouse) and amphotericin B (20 μg per mouse), splenomegaly was by 26% lower in comparison with controls. As low as 100 μg doses of (2'-5')ApApA per mouse (4 mg/kg) exhibited a significant reduction of splenomegaly when administered to the infected mice daily for 15 days in combination with amphotericin B (Table 1, experiment III). A very high antiviral effect of (2'-5')ApApA in Rauscher murine leukaemia was demonstrated by 72% suppression of viraemia as measured by reverse transcriptase activity in sera of treated and control animals.

Inhibition of RMLV replication in the transformed spleen cells was clearly demonstrated by electron microscopic analysis of ultrathin sections of spleen cells in the control group (Fig. 1) and groups treated with (2'-5')-ApApA (1 mg per mouse daily on 5 consecutive days) (Fig. 2), and the group treated with 5 doses of (2'-5')-oligoadenylate simultaneously with amphotericin B (Fig. 3). Analysis of at least 20 ultrathin sections in each group revealed a very significant reduction (about 95%) in the number of virions in the spleen cells of treated animals as compared with the controls.

Discussion

The results obtained demonstrate a much higher antiviral effect of exogenous (2'-5')ApApA on RMLV than that observed with IFN and RNA tumour viruses (Friedman and Ramseur, 1974; Billiau, 1977; Billiau *et al.*, 1978). We suppose that in the retrovirus-transformed cells the induction of (2'-5')-oligoadenylate synthetase by exogenous IFN may be impaired by some yet unknown molecular mechanism. Moreover, this hypothesis should be proved experimentally.

Since the dephosphorylated oligonucleotide (2'-5') ApApA did not activate RNase F in a cell-free system (Kerr and Brown, 1978), it could act either

by being phosphorylated at the 5'-OH after entering the cell, or by some yet unknown mechanism. The finding of Kimchi *et al.* (1981) that a free 5'-OH in the (2'-5')ApApA was essential for antimitogenic activity of the oligonucleotide in 3T3 fibroblasts supports the hypothesis that it was phosphorylated to (2'-5')pppApApA. The destruction of viral RNA by (2'-5') pppApApA- activated RNase F in the transformed spleen cells is very probably the main molecular mechanism of the antiviral action of (2'-5')ApApA in Rauscher murine leukaemia.

The metabolism of (2'-5')ApApA in vivo is not yet known, but probably a great part of the oligonucleotide may be degraded to (2'-5')ApA and to 5'-AMP. The dimer (2'-5')ApA may inhibit in vivo the catabolizing enzyme 2'-phosphodiesterase, as has been shown in a cell-free system (Kimchi *et al.*, 1981*b*). On the other hand, 5'-0'-monophosphoryl-derivative, (2'-5')pApApA, antagonized the protein synthesis inhibitory effect of (2'-5')pppApApA by preventing activation of RNase F which degraded mRNA (Torrence *et al.*, 1981). In cells deficient in RNase F, neither cellular protein nor DNA synthesis were inhibited by (2'-5')pppApApA (Panet *et al.*, 1981).

Because very low concentration (nanomolar) of the (2'-5')pppApApA is sufficient to activate RNase F (Williams and Kerr, 1978; Baglioni *et al.*, 1981), the amount of (2'-5')ApApA converted in vivo to (2'-5')-pppApApA is probably very small but sufficient to mediate the antiviral effect of exogenous (2'-5')ApApA.

The difference between the anticellular and antiviral effects of (2'-5')ApApA in the RMLV-infected mouse spleen in vivo is striking. Whereas inhibition of splenomegaly by (2'-5')ApApA (1 mg per mouse, 5 doses) was only 17% (Table 1), inhibition of virus replication was much higher, more than 95% (Fig. 1 and 2). The infectivity of virions present in the spleen cells of the (2'-5')ApApA-treated animals may also be reduced. This possibility deserves further experimental research.

Oligonucleotide (2'-5')pppApApA and the cordycepin analog (2'-5') ppp3'dA(p3'dA)$_2$ have been reported to prevent the transformation of human lymphocytes after infection with Epstein-Barr virus (Doetsch *et al.*, 1981). Because the (2'-5')ApApA is not toxic in vivo (we have observed that a dose as high as 10 mg per mouse given i.p., i.e. 400 mg/kg, was well tolerated by the animal), the use of this synthetic oligonucleotide seems to be indicated in the chemotherapy of tumours induced by retroviruses and DNA tumour viruses.

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Explanation of Electron Micrographs (Plates LI—LII):

Fig. 1. Ultrathin section of mouse spleen infected with RMLV (21 days p.i.); untreated animal (Tabl 1, exp. I, group 1). Numerous mature virions and budding particles are present in the transformed cell.

Fig. 2. Electron microscopic picture of leukaemic spleen from a mouse infected with RMLV and treated with 5 i.p. injections of (2'-5')ApApA (1 mg per mouse) during the first 5 days after infection (Tab. 1, exp. I, group 2). The spleen was removed 21 days p.i. A few virions of type A can be seen.

Fig. 3. Electron microscopic picture of the spleen of a mouse infected with RMLV and treated with 5 i.p. injections of (2'-5')ApApA (1 mg per mouse) and amphotericin B (20 μg in 0.2 ml saline per mouse) (Tab. 1, exp. I, group 3). The spleen was taken 21 days p.i. Virus replication was nearly completely inhibited in this group of animals. One virus particle of type A is present in this particular microscopic field only.