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In Vitro and *In Vivo* Antitumor Effect of Dimerized Ribonuclease A*

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Abstract—*A cross-linked dimer of bovine pancreatic RNase A blocks the proliferation of tumoral cells when it is added at a concentration of* 10 μg/ml *to their culture medium. The effect is not related to the enzyme activity towards RNA; it is also observed* in vivo *when the dimer is injected intramuscularly in the vicinity of solid tumors induced in rats by hepatoma cells: treated rats show a marked reduction in tumor development or no tumor. Furthermore, the rats having responded to the treatment with the dimer do no longer develop tumors when they are submitted again to injections of hepatoma cells.*

The action of the ribonuclease dimer has been tested on other cell lines in culture and compared with similar reports published on the naturally occurring dimer, bovine seminal RNase. Four different types of tumoral cells in culture have been tested; 3 of those interiorize the dimer and their proliferation is blocked, teratoma cells however do not fix a large amount of the dimer and are not affected; 3 normal cell lines tested (rat and human fibroblasts, macrophages) do not fix an important amount and are not affected by the ribonuclease dimer. This cytostatic effect is also compared to that of other similar basic proteins which are found less specific and less cytostatic but more toxic.

INTRODUCTION

IT HAS previously been reported that a dimer of pancreatic RNase A obtained by cross-linking with dimethyl suberimidate is cytostatic when added to the culture medium of tumor cells [1]; this has been confirmed *in vivo* in leukaemic AKR mice [2]. RNase A monomer was also shown to decrease the growth of malignant tumors when injected at relatively high doses to animals [3–6]; however since the RNase dimer is fixed in larger amounts by tumor cells than the monomer [1, 7], a greater inhibition of neoplastic growth could be expected at a lower concentration for the dimer than for the monomer.

The present study was undertaken to investigate the antitumoral properties of dimeric ribonuclease A *in vivo* and *in vitro* and to compare the two effects; some of the results have been presented in abstract form [8]. Dependence of the effect on the enzymatic properties of the dimer and on the amount taken up by the cancerous cells has been investigated and compared to normal cell lines.

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MATERIALS AND METHODS

RNase dimer

Ribonuclease dimer was prepared by cross-linking beef pancreas RNase A with dimethyl suberimidate [9, 10]. For inactivation of the enzyme, the dimer was alkylated by a modification of the procedure described by Crestfield *et al.* [11] for ribonuclease A. Ribonuclease dimer (50 mg) was dissolved in 4 ml of 0.02 M Na acetate buffer, pH 5.5. Iodoacetic acid (200 mg), was added to the dimer solution. Reaction was allowed to proceed for 3 hr at room temperature and the preparation was then submitted to gel filtration on Sephadex G-100. When carboxymethylated in this manner, the dimer retained 8% of its activity toward yeast RNA. Another preparation was carboxymethylated in 10% iodoacetic acid for 8 hr; in this case the dimer had 2% of residual RNase activity toward yeast

RNA. Ribonuclease determinations were performed as described before [12].

Cell cultures

Cell proliferation was measured by counting the cells under the microscope with a Burker cell; protein concentration was also routinely measured on cell pellets.

Hepatoma tissue culture cells (HTC) derived from Morris hepatoma 7288C induced in Buffalo rats, were grown at 37°C in suspension according to Samuels and Tomkins [13]. The culture was started at a concentration of 4×10^5 cells per ml in presence or absence of RNase dimer.

Sarcoma cells (SR), derived from a rat tumor induced by a Schmidt–Ruppin strain of Rous sarcoma virus (ATCC-CCL 47) were grown in modified Eagle's culture medium [14], used with 20% foetal calf serum. Proliferation was measured after 40 hr on cell cultures started at a density of 10^5 cells/cm^2 and grown normally or in presence of 50 μg/ml RNase dimer.

L1210 leukemia cells were obtained from an experimental mouse leukemia transmitted intraperitoneally. Cells were grown in suspension in the R.P.M.I. medium 1640 supplemented with 10% foetal calf serum. The proliferation was measured after 40 hr on cells started at a concentration of 5×10^5 cell per ml and grown in presence or absence of RNase dimer.

Teratocarcinoma cells from line PCC4 azal, a permanent cell line derived from the transplantable teratocarcinoma OTT 6050 [15], were cultured in the Dulbecco modification of Eagle's medium supplemented with 15% foetal calf serum in an atmosphere of 12% CO_2. Proliferation was measured after 4 days.

Rat embryo fibroblasts were grown according to Tulkens *et al.* [14]. Cultures were started at a density of 5×10^4 cells/cm^2 in Falcon flasks with or without ribonuclease and the cells detached with 2.5 g/l trypsin were harvested.

Human skin fibroblasts were cultivated according to Leroy *et al.* [16]. Proliferation was measured on cultures started at a density of 5×10^4 cells/cm^2 for rat fibroblasts.

Rabbit bone marrow isolated from femurs was cultured in a liquid medium in the presence of high concentrations of serum and eventually in presence of ribonuclease dimer but without other exogenous stimulating substances [17] in order to have a specific proliferation of macrophages after 4–6 days of culture; the cell proliferation was measured after 7 days.

Transplantation of tumor cells in rats and measurement of tumor growth

HTC cells were harvested from their culture medium, washed 2 times with 0.9% NaCl. Cells, 2×10^6 in 1 ml NaCl, were injected in the left hind leg of male Buffalo rats. The rats develop a macroscopic tumor within 10 days; female rats are however, more resistant to those tumoral cells and a higher cell concentration is needed to have reproducible solid tumors. The animals were examined every week for tumor growth and the diameter of their legs at the tumor level was measured in two perpendicular directions. For the evaluation of tumoral growth, tumor section was considered as an ellipse; the following formula was used for calculation: tumoral section $= t_1 t_2 \pi/4 - c_1 c_2 \pi/4$, where t_1 and t_2 are the perpendicular axes of the tumoral left leg and c_1 and c_2 the perpendicular axes of the control right leg.

Materials

Ribonuclease A (Type I-A), yeast RNA (Type II-S), cytochrome c (Type III from horse heart), histone (type II from calf thymus), lysosyme and poly-L-lysine (Type III) were from Sigma Chemical Co. (St. Louis, Mo.); dimethyl suberimidate dihydrochloride was from Pierce Chemical (Rockford, III.); iodoacetic acid from Merck A. G. (Darmstadt).

RESULTS

In vitro *effect of RNase dimer*

When proliferation of HTC cells in their culture medium was blocked by RNase dimer (Fig. 1), the concentration dependency of the effect of the dimer on cell multiplication was identical whether the dimer was enzymatically active or was inactivated by reaction with iodoacetate. The cytostatic properties of the cross-linked dimer are therefore independent of its enzymatic activity. The dimer was cytostatic against hepatoma cells, leukemia L1210 cells, and sarcoma cells; no effect could be observed on so-called normal cell lines like rat embryo fibroblasts or human skin fibroblasts and rabbit bone marrow macrophages (Table 1). One cancerous cell line however, the teratoma pluripotent cells, was found resistant to the dimer; the effect on leukemic

cells was less pronounced than on hepatoma cells. As shown before [1], we have observed that cells blocked by the dimer fixed much more RNase (about 5 μg/mg protein) than all the cells that we have found resistant to it (less than 0.5 μg/mg protein).

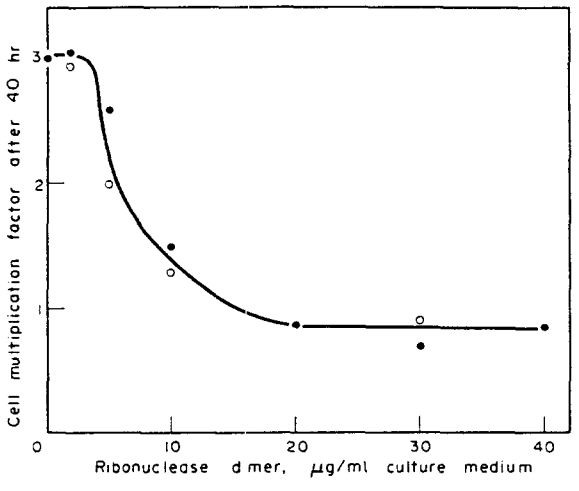

Fig. 1. Cytostatic effect of dimerized RNase in vitro. *The cross-linked dimer of RNase A enzymatically active* (●) *or* 90% *inactivated with iodoacetic acid* (○) *was added at different concentrations to the culture medium of HTC cells grown in suspension in the logarithmic phase. The cell concentration was measured at the microscope at time* 0 *and* 40 hr *using a Burker cell. The cell multiplication factor was determined as* 1 *in absence of duplication.*

In vivo *effect of RNase dimer on tumors*

An antitumor activity *in vivo* has been described for the cross-linked dimer injected i.p. to mice inoculated i.p. with S180J ascitic sarcoma cells [2]. We have found that the enzymatically active or inactivated dimer was antitumoral when injected in the vicinity of solid tumors induced in rats. Buffalo rats inoculated i.m. (in the left hind leg) with a suspension of 2×10^6 HTC cells developed rapidly a solid tumor, this tumor grew exponentially as measured by the increase in tumor section, and necrosis followed by death occurred after two months (Fig. 2). If RNase dimer (active or inactivated) was injected i.m. to the rats (2 mg/animal 3 times at 3 day interval in the vicinity of the tumor), a marked reduction in tumor development could be observed; RNase A had no effect at this concentration. The rats treated with the dimer showed only small tumors after 50 days (11 out of 15 rats had no detectable tumor), while the rats treated with RNase A monomer had macroscopic tumors at the same time (19/20 rats). These results obtained *in vivo* thus confirm that enzyme activity is not required for the antitumoral activity; however dimerization is required.

Those observations can be compared favorably with the effects observed *in vivo* by

Table 1. Cytostatic properties of the cross-linked dimer of RNase A

Cell type	time (hr)	Cell proliferation (Cell number/10^5 × ml) Control cells	+RNase dimer (50 μg/ml of culture medium)
Hepatoma	0	35	35
HTC Morris	20	59	30
	40	87	26
Sarcoma	0	10	10
Smidt-Ruppin	20	19	13
	40	39	13
Leukemia	0	50	50
L 1210	20	130	65
	40	240	88
Teratoma	0	10	10
pluripotent cells	100	100	96
Fibroblasts	0	5	5
Human skin	20	8.6	8.3
	40	13.7	13.5
Fibroblasts	0	5	5
Rat embryo	20	11	11
	40	16	15.5
Macrophages	0	5	5
Bone marrow	150	280	275

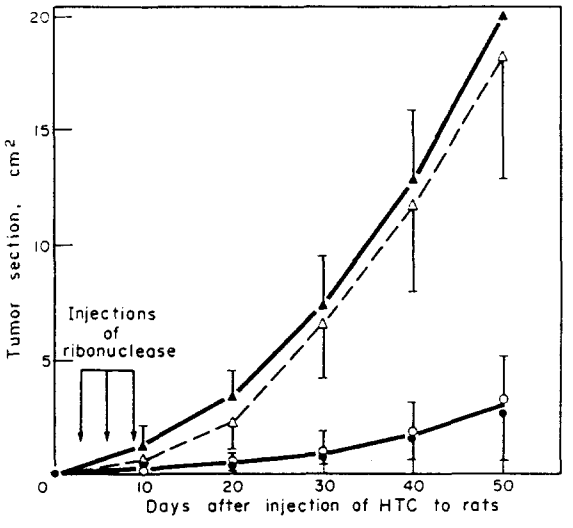

Fig. 2. Effect of dimerized RNase on solid tumors in vitro. *Buffalo rats were injected i.m. in one leg with* 2×10^6 *HTC cells at* day 0; *they were then treated i.m. in the vicinity of the tumor at* day 3, 6, 9 *with* 1 ml 0.9% NaCl (▲), *with* 2 mg *RNase A in* 1 ml 0.9% NaCl (△), *with* 2 mg *of enzymatically active ribonuclease dimer* (●), *or with* 2 mg *of* 90% *inactivated dimer* (○). *The size of the tumors was then measured weekly. The curves presented are each the mean and standard deviations of the values observed for* 10 *rats; when overlapping occurs between two curves, the standard deviations are only presented on the upper or the lower part of the median.*

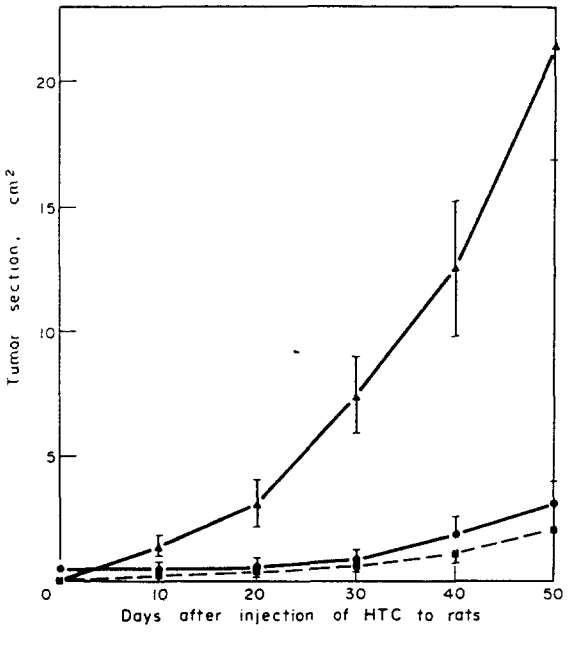

Fig. 3. Protective effect against tumor development obtained by pretreatment with irradiated HTC cells or HTC cells and RNase dimer. Buffalo rats were pretreated once 6 weeks *before inoculation by i.m. injection of* 0.9% NaCl (▲), *or of* 2.10^6 *HTC cells killed by* 100 min *irradiation at* 5000 rad (■), *or of* 2.10^6 *HTC cells; in this last case, this injection was followed by* 3 *weekly injections of* 2 mg *of dimerized ribonuclease* (●). *At* day 0, 2.10^6 *viable HTC cells were then injected and the experimental procedures followed were similar to those of Fig. 2. Each curve presented is the mean and standard deviations of the values obtained for* 5 *rats.*

others using the bull seminal RNase dimer. Matousek *et al.* [18–22] observed a similar effect on different animal models, with however a higher toxicity; the seminal RNase dimer inactivated by carboxymethylation kept its antitumor effect and was less toxic [23].

It was of interest to test if animals having received a first treatment with ribonuclease dimer were still able to develop tumors after a new injection of HTC cells. Buffalo rats were therefore injected with HTC cells and treated normally by the dimer; after 60 days the rats had no macroscopic tumors and were injected again with a suspension of 2.10^6 HTC cells i.m. Those rats were not submitted to a new RNase treatment and however, they did not develop solid tumors anymore in contrast to control rats (Fig. 3). The resistance acquired against HTC cells can be compared with the one that we have obtained with rats pretreated with HTC cells killed by irradiation (Fig. 3). The cells irradiated did not induce the formation of solid tumors when injected i.m. to rats; they did protect the rats against new injections of viable HTC cells; indeed, the rats injected again after 2 months with 2.10^6 HTC cells did not develop tumors.

Comparison with other similar basic proteins

As shown by the previous observations, the cytostatic action of RNase dimer is independent of the enzyme activity but is related to its dimeric structure. Other basic proteins of similar molecular weight have therefore been tested for their cytostatic effect on HTC cells and their uptake; the results are presented in Table 2. It can be seen that the dimer inactivated up to 90% (still positively charged) kept its effect on cell proliferation and its high uptake. However, the dimer inactivated up to 98% (± 2) with iodoacetic acid lost its high uptake and the cytostatic effect; probably due to modifications of some lysines in addition to the carboxymethylhistidines. Higher ribonuclease polymers (mostly tetramers) obtained by cross-linking were well taken up by the cells, but without cytostatic effect, RNase A itself being poorly taken up and without influence. The histone preparation tested (showing 2 protein bands of mol. wt 12,000 and 28,000 respectively in S.D.S. gel electrophoresis) was well endocytized and effective *in vitro*; the preparation was however toxic towards normal fibroblasts at a concentration of 50 μg/ml and had no protective effect *in vivo*. Cytochrome c, either monomer or dimerized by cross-linking with dimethyl

Table 2. Cytostatic effect of various compounds on HTC cells in vitro *and* in vivo *on solid tumors induced in rats by HTC*

Compounds	*In vitro* (Mean of 3 experiments) % Inhibition of cell multiplication after 24 hr compared to control cells	Uptake μg/mg protein after 24 hr	*In vivo* % Inhibition of tumor development after 1 month
Cross-linked dimer of RNase A	100 ± 16	6.1 ± 0.3	89 ± 8 (20 rats)
Dimer 91% inactivated with iodoacetate	98 ± 13	5.7 ± 0.4	90 ± 7 (10 rats)
Dimer 98% inactivated with iodoacetate	19 ± 12	0.5 ± 0.2	21 ± 5 (10 rats)
Polymerized ribonuclease A	31 ± 18	12.2 ± 2.5	20 ± 3 (5 rats)
Ribonuclease A	22 ± 15	0.5 ± 0.1	8 ± 6 (10 rats)
Histone	85 ± 16	4.8 ± 0.6	0 ± 3 (10 rats)
Cytochrome c	0 ± 10	0.7 ± 0.4	0 ± 1 (5 rats)
Cytochrome c dimerized	16 ± 14	1.0 ± 0.8	—
Poly-lysine	77 ± 16	—	—
Lysozyme dimerized	73 ± 15	8.2 ± 2.0	—
Lysozyme monomer	8 ± 4	0.9 ± 0.3	—

*For the *in vitro* experiments, the different compounds were added at concentration of 50 μg/ml to the culture medium of HTC cells. For the *in vivo* experiments, Buffalo rats were injected i.m. in the vicinity of the tumor with 2 mg of the different compounds at day 3, 6, 9 after the injection of 2×10^6 HTC cells i.m. to the rats. Results are given as means ± standard deviations.

suberimidate had a low uptake and no effect at all at this concentration. Poly-lysine was cytotoxic *in vitro* but also very toxic to all the cell types tested whether tumoral or not. The effect of dimerized lysozyme could be more promising since it is well taken up and has a cytostatic effect and low toxicity; it merits further investigation. The dimerized ribonuclease was up to now the only basic compound tested to be cytostatic toward tumoral cells *in vitro*, and *in vivo* without loss of body weight or major toxicity even when injected at a dose of 25 mg per rat.

DISCUSSION

The effects of RNase A monomer on the neoplastic growth have been controversial; Ledoux [5, 6], Bergel [3], Graffi and Arnold [4] found an effect while de Lamirande [24] found no effect on Ehrlich tumors. The concentration required for the effect *in vivo* was high: 25 mg/mouse. In our experiments, the RNase dimer was effective at much lower concentration (100-fold lower) and could lead to a complete regression of tumors when injected in their vicinity. The possibility of an immunologic stimulation of the rats by the ribonuclease dimer itself has to be envisaged, but the dimer injected to the rats before any injection of tumoral cells had no protective effect. The inhibitory action of RNase A dimer on cell proliferation has already been demonstrated *in vitro* [1] and *in vivo* [2], but the results reported here show for the first time a good agreement between the two systems. It has been shown by others that the naturally occurring dimer of RNase, bovine seminal ribonuclease [25], affects cytostatically the testicular and embryonic tissues in mice and other species [19]; it inhibits the proliferation of leukemic and HeLa cells and causes the reduction of Walker carcinosarcoma, Sajdel hepatoma and Crocker tumors *in vivo* [18–22]. Bovine seminal ribonuclease can also block the growth of polyoma transformed BHKT 6 cells, while it has no effect on normal BHKT 6 hamster cells [26]. Those effects are in good agreement with the results obtained with the synthetic dimer. Bovine seminal RNase is also antitumoral but presents apparently a higher toxicity on normal cells than the synthetic dimer.

The loss of ribonuclease activity has no consequence on the antitumor properties; this has been confirmed after carboxymethylation of RNase dimer or seminal RNase [23]. The modification was not accompanied by a loss of antitumor activity for the dimer inactivated up to 8%, but this inactivation did lower the toxicity of seminal RNase [23].

The binding of bovine seminal RNase to the surface of tumoral cells has been shown in mouse leukemia, Walker tumors and hepatoma; the absorption activity of the tumors for RNase was higher than the one of other body organs [5–7, 20]. This high affinity can explain the specific action of ribonuclease on tumors. Other basic compounds were expected to have similar properties since the effects

observed were not related to enzyme activity; the compounds that we have tested were however not very promising mostly due to their toxicity. Histones and poly-lysine have been shown to enter tumor cells rapidly and in large quantity and to destroy the viability of the cells [27]; this property seems however not to be specific for cancerous cells; poly-lysine like other polycations is toxic at a concentration equal or higher than 50 μg/ml, it binds to the cell surface [28, 29] and causes lysis of the membrane. Further Mayhew *et al.* [28] have shown that low molecular weight poly-lysine is much less toxic than high molecular weight poly-lysine which alters at low concentration the permeability of ELD cell membranes. This confirms that the effects of basic compounds on cells are dependent of their molecular weight.

An important problem encountered for the *in vivo* tests is the accessibility of the target cells to the dimer; in most cases RNase had to be injected in the growing tumor or in its vicinity in order to be effective.

At the present stage, the RNase dimer can be considered as a possible addition to the known models of cytostatics more effective on tumors than on the host cells; it could be used as a model for potential drugs or as carrier for drugs killing tumor cells but damaging the healthy ones as little as possible.

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