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Inhibition of tumor cell proliferation by dimerized ribonuclease

(antimitotic effect/cytotoxicity/endocytosis/lysosomes/cancer)

JACQUES BARTHOLEYNS AND PIERRE BAUDHUIN

Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, Avenue Hippocrate 75, B-1200 Brussels, Belgium

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ABSTRACT **A cross-linked dimer of pancreatic ribonuclease A (ribonucleate 3′-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22), at a 10 mg/liter concentration, blocks proliferation of tumor cells. The protein retains this ability after inactivation by iodoacetate. The cytostatic effect of ribonuclease preparations on various cell lines correlates well with their rate of uptake: for example, monomeric ribonuclease A is much less effective and is taken up into the cells 10 to 15 times more slowly. Cell fractionation studies on hepatoma cells indicate accumulation of the dimer in the lysosomal system. Ribonuclease dimer induces a labilization of the lysosomes when added to cell homogenates, raising the possibility that its antitumoral effect may be mediated by endocytosis and lysosomes.**

Various reports have suggested that pancreatic ribonuclease A (ribonucleate 3′-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22) can penetrate intact animal cells (1–6). High concentrations of ribonuclease A have been reported to inhibit tumor growth when the enzyme is injected into animals, and to interfere with ascites cell multiplication in culture (5–7).

A synthetic dimer of bovine pancreatic ribonuclease A has been prepared by cross-linking (8, 9). It has enzymic properties similar to those reported for the naturally occurring ribonuclease dimer from bovine seminal plasma (10, 11), which has been shown to exert a cytostatic effect (12, 13). The present research was initiated to determine whether the synthetic dimer is cytostatic to cells grown in culture. Binding of ribonuclease to the cell membrane and its endocytosis by different cell lines have been studied and compared with its cytostatic activity.

MATERIALS AND METHODS

Cell Cultures and Homogenization. Hepatoma tissue cells (HTC) are derived from Morris hepatoma 7288 C induced in Buffalo rats. The cells were grown at 37° in suspension according to Samuels and Tomkins (14). Cells in logarithmic growth were harvested, washed three times with 0.15 M NaCl, and sedimented at 800 rpm for 10 min (International centrifuge model PR-J., International Equipment Co., Needham, Mass.). Cells at a concentration of 5×10^7/ml were then homogenized in 0.25 M sucrose by 30 strokes of the tight pestle of a Dounce homogenizer (Kontes Glass Co., Vineland, N.J.).

Rat embryo fibroblasts were grown according to Tulkens *et al.* (15). Cultures were started at a density of 5×10^4 cells per cm^2 in Falcon flasks and the cells were harvested after 48 hr. After washing three times with phosphate-buffered saline (0.15 M NaCl, 2.7 mM KCl, 3 mM Na_2HPO_4–KH_2PO_4, pH 7.4), cells were detached from the flasks with 0.5 mM EDTA, suspended and spun down at 800 rpm for 10 min. They were homogenized in 7 ml of 0.25 M sucrose containing 1 mM EDTA, pH 7.4, by six strokes of the tight pestle of a Dounce homogenizer.

Human skin fibroblasts were cultivated according to Leroy *et al.* (16). Culture conditions, harvesting, and homogenization were as described for rat fibroblasts, except that cells were detached with 2.5 g/liter of trypsin.

Sarcoma cells (SR) were derived from a rat tumor induced by a Schmidt-Ruppin strain of Rous sarcoma virus. Our strain was issued by the American Type Culture Collection (ATCC-CCL 47). Eagle's culture medium (17) was used with 20% calf serum. Culture conditions, cell harvesting, and homogenization were as for HTC cells.

L-1210 leukemia cells were obtained from an experimental mouse leukemia transmitted intraperitoneally (18). Cells were grown in suspension in medium 1640 from Roswell Park Memorial Institute, Buffalo, N.Y. (19). Harvesting and homogenization were carried out as described for HTC cells.

Fractionation of HTC Cells. For analytical studies, homogenates were prepared from 5×10^8 cells. After homogenization, fractionation by differential centrifugation was performed according to the scheme of de Duve *et al.* (20), to yield a nuclear fraction N, a heavy mitochondrial fraction M, a light mitochondrial fraction L, a microsomal fraction P, and a final supernatant S.

Ribonuclease Dimer. The synthetic dimer was prepared by cross-linking with dimethyl suberimidate as described by Bartholeyns and Moore (8). Specific ribonuclease activity of the dimer was 3000–4000 units/mg of protein, depending on the preparation. For inactivation of the enzyme, the dimer was alkylated by a modification of the procedure described by Crestfield *et al.* (21) 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), dissolved in 2 ml of water and adjusted to pH 5.5 with NaOH, was then added to the dimer solution. Reaction was allowed to proceed for 200 min at room temperature. The preparation was then submitted to gel filtration on Sephadex G-100. When carboxymethylated in this manner, the dimer had only 6% of residual activity toward yeast RNA.

To obtain labelled inactivated dimer, iodo-[2 − ^{3}H]acetic acid was used for alkylation. Fully active dimer was labeled using tritiated acetic anhydride. In this case, ribonuclease dimer (50 mg) was dissolved in 15 ml of 0.02 M Na phosphate buffer, pH 8.0. Tritiated anhydride (1 mg), dissolved in 1 ml of dioxane, was then added to the dimer solution. Reaction was allowed for 60 min at 20°. The preparation was then submitted to gel filtration on Sephadex G-25.

Biochemical Assays. Ribonuclease determinations were performed as described by Bartholeyns *et al.* (22). One unit of enzyme activity corresponds to the release in acid-soluble form of 1 μmol of mononucleotide equivalent per min.

Cytochrome oxidase (EC 1.9.3.1) was measured as described by Appelmans *et al.* (23) except that 2 g/liter of dig-

Abbreviations: HTC cells, hepatoma tissue cells; SR cells, Schmidt-Ruppin sarcoma cells.

itonin was added to the preparation; alkaline phosphodiesterase I (EC 3.1.4.1), according to Beaufay *et al.* (24); acid phosphatase (EC 3.1.3.2), as described by de Duve *et al.* (20); catalase (EC 1.11.1.6), according to Baudhuin *et al.* (25); protein by the method of Lowry *et al.* (26), and *N*-acetyl-β-glucosaminidase (3.2.1.30) according to Sellinger *et al.* (27). When free activity was measured, 0.25 M sucrose was included in the reaction medium and detergent was omitted; the incubation time was kept at 10 min in order to minimize damage to particles.

Materials. Tritium-labelled acetic anhydride (49 mCi/mg) and iodo-[2-^{3}H]acetic acid (25 mCi/mg) were from the Radiochemical Centre Ltd., Amersham. Ribonuclease A (Type I-A) and yeast RNA (Type II-S) were from Sigma Chemical Co. (St. Louis, Mo.); dimethyl suberimidate dihydrochloride was from Pierce Chemical (Rockford, Ill.); iodoacetic acid, from Merck A.G. (Darmstadt); and trypsin from Difco Laboratories (Detroit, Mich.).

RESULTS

Cytostatic effect of ribonuclease dimer on hepatoma cells

Fig. 1 illustrates the effect of various ribonuclease preparations added at a concentration of 50 mg/liter to the culture medium of HTC cells. Control cells multiply normally, with a doubling time of approximately 24 hr. Cell proliferation is inhibited only slightly by ribonuclease A, completely by ribonuclease dimer, whether enzymically active or alkylated. A cytotoxic effect could be observed after 20 hr, the cell number decreased, and some cell lysis occurred. We have verified that no apparent inhibition of DNA synthesis occurs in presence of dimer; measurement of thymidine incorporation showed a constant value per mg of protein up to 20 hr after addition of RNase; incorporation then dropped abruptly.

This inhibition of cell multiplication was observed at concentrations as low as 10 mg/liter of either active dimer or dimer inactivated with iodoacetate. These preparations were thus far more effective than RNase A, which had to be added at 500 mg/liter to produce a similar effect. Moreover, these experiments indicate that the enzyme activity of the dimer is not essential for its cytostatic effect, since inactivated dimer was equally effective.

Uptake of RNase by hepatoma cells

HTC cells were incubated for 1 hr at 0° or 37° in the presence of 50 mg/liter of RNase dimer or monomer, and then washed three times with 0.15 M NaCl. At 0° the amount of dimer retained by the cells was about 15 times the amount of monomer retained. Retention of dimer was 20% lower at 37°, whereas that of the monomer was twice as high as at 0°, although still only one-sixth of that of the dimer. If the washed cells were then grown again in suspension, duplication was blocked for cells incubated either at 0° or at 37° with RNase dimer. On the other hand, if washing was performed with whole culture medium, the amount of RNase remaining on the cells was much smaller and no more cytostatic effect could be detected. Fixation of RNase on the cell membrane, therefore, did not in itself induce a rapid process that blocked duplication.

Uptake of RNase was followed during the time course of cell culture (Fig. 2). RNase dimer was taken up by the cells 20 times faster than RNase A, at least during the first 6 hr of culture. Although the small residual activity of the dimer treated with iodoacetate did not allow an accurate comparison of the rate of uptake of the two forms of dimer, the results presented in Fig. 2 are consistent with an accumulation of inactivated dimer similar to that observed for the active dimer. This point was further verified using labeled dimer preparations (see also Table 1).

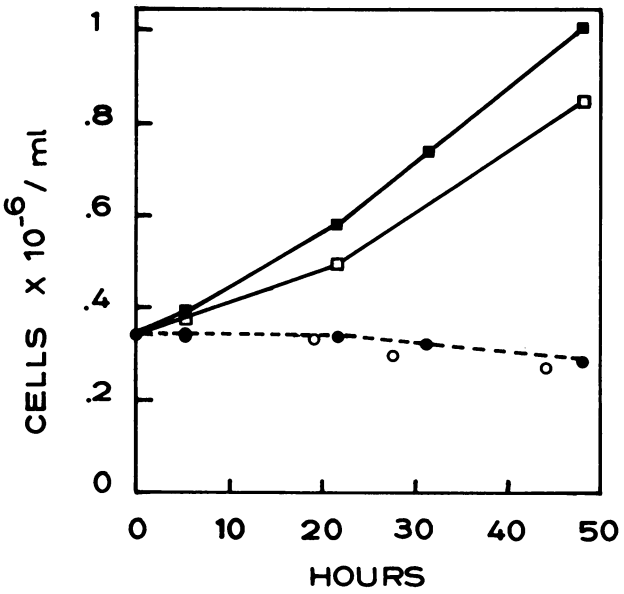

FIG. 1. Cytostatic effect of RNase dimer. HTC cells at concentration of 3.5 × 10^5 cells per ml were grown in suspension at 37° in four different conditions: without any addition (■), in presence of 50 mg/liter of RNase A (□), 50 mg/liter of RNase dimer (●), and 50 mg/liter of RNase dimer alkylated with iodoacetate (○). The cell concentration was estimated under the microscope with a Fuchs-Rosenthal cell.

Relation between uptake of RNase and cytostatic effect

The uptake of RNase A, RNase dimer, and alkylated dimer was compared for different cell types (Table 1.) Cell multi-

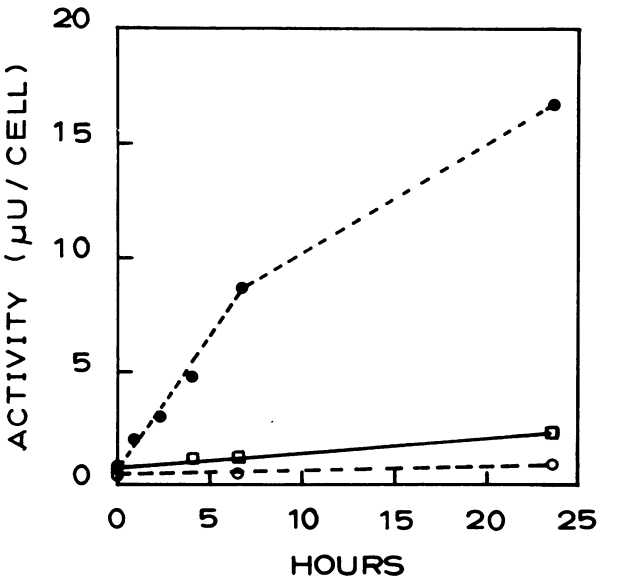

FIG. 2. Uptake of RNase by HTC cells. Cells were grown in the same conditions as in Fig. 1: in presence of 50 mg/liter of RNase A (□), 50 mg/liter of RNase dimer (●), or 50 mg/liter of alkylated RNase dimer (○). Aliquots of 5 ml of cell suspension were taken from each spinner at different intervals; the cells were spun down, washed three times with 0.15 M NaCl, and homogenized in 1 ml of 0.2% Triton X-100. Ribonuclease activity was then measured in each case and expressed as microunits per cell.

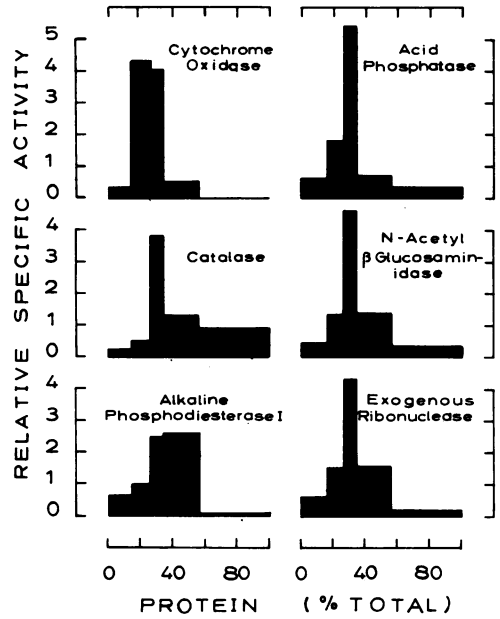

FIG. 3. Distribution patterns of enzymes after fractionation by differential centrifugation. HTC cells were incubated for 2 hr in the presence of RNase dimer, and then grown for 3 hr in culture after resuspension in a RNase-free medium. The cells were then homogenized, and subdivided into five fractions: N, M, L, P, S, according to the scheme of de Duve *et al.* (20). Ordinate: mean relative specific activity of fractions. Abscissa: fractions are represented by their relative protein content, cumulatively from left to right, in the order in which they are isolated. Cytochrome oxidase is a marker enzyme for mitochondria, catalase for peroxisomes, alkaline phosphodiesterase I for plasma membranes, acid phosphatase and *N*-acetyl-β-glucosaminidase for lysosomes (28). Recoveries from the homogenate ranged between 81 and 102%.

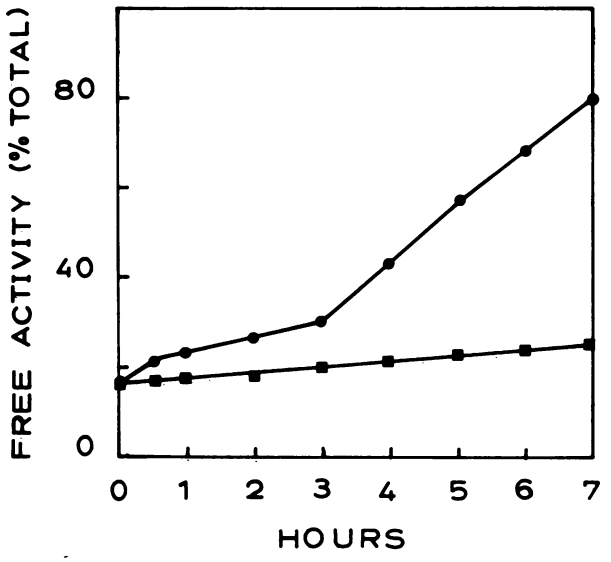

FIG. 4. Labilization of lysosomes *in vitro* by dimerized ribonuclease. Homogenates of HTC cells (1×10^6 cells per ml) were incubated at 37° in 0.25 M sucrose, 0.01 M phosphate buffer, pH 7.4, in the presence (●) or absence (■) of 100 mg of RNase dimer per liter. Free and total *N*-acetyl-β-glucosaminidase activities were measured at regular intervals on aliquots of the homogenate.

plication was measured at the same time during at least 50 hr in each case. A correlation can be observed between ribonuclease uptake and cytostatic effect. It was found that increasing the RNase A concentration in the culture medium up to 500 mg/liter induces an uptake by HTC cells similar to that observed with the dimer at 50 mg/liter. At this high concentration, RNase A exerts a cytostatic effect. The difference in effectiveness observed between RNase A and the dimer can, therefore, probably be largely explained by a difference in uptake.

The results presented confirm also that the enzyme activity is not an essential factor for the antimitotic activity. Ribonuclease does not appear to act by breaking down some cellular RNA: uptake and cytostatic effects are the same, whether the dimer is inactivated or not.

Another important point shown in Table 1 is that the high specific uptake of the dimer was observed with three distinct types of tumor cells, but not with fibroblasts. More extensive observations on various cell types are necessary in order to indicate the degree of tumor cell specificity of the dimer. It is, however, striking that the ribonuclease dimer is devoid of toxicity in mice, even when injected at high doses (25 mg per animal), suggesting that a number of normal cell

Table 1. Relation between the amount of RNase taken up in 24 hr by various cell types and the cytostatic effect

	No addition	RNase A monomer		RNase dimer		94% Inactivated dimer	
Cell types	Multiplication factor*	Uptake, μg/mg†	Multiplication factor*	Uptake, μg/mg†	Multiplication factor*	Uptake, μg/mg†	Multiplication factor*
Hepatoma cells (HTC)	1.85	0.50	1.72	6.10 5.60‡	0.97	4.80‡	0.89
Sarcoma cells (SR)	2.00	0.30	2.01	3.20	1.00	—	0.76
Leukemia cells (L-1210)	3.30	—	3.20	1.60	1.58	—	—
Rat fibroblasts	2.30	0.40	2.20	0.40	2.50	—	2.35
Human fibroblasts	1.45	0.50	1.44	0.50	1.48	—	—

* The multiplication factor is defined as the ratio of the number of cells counted after 24 hr of culture to the number present at the beginning of the experiment. This factor will be 1 if cell duplication is totally blocked and lower than 1 if there is cytotoxicity.

† Cells in culture were incubated 24 hr at 37° in presence or in absence of 50 mg of RNase per liter of culture medium. RNase uptake, expressed in μg/mg of cell protein, was determined by the increase in RNase activity of the cells, except when indicated otherwise.

‡ Labeled dimer was used for determination of RNase uptake.

types share with fibroblasts a relatively low sensitivity to its cytotoxic effect.

Fate of the ribonuclease dimer after cellular uptake

Homogenates of HTC cells exposed to RNase dimer were fractionated by differential centrifugation. Hepatoma cells were kept in culture for 2 hr in the presence of ribonuclease dimer (50 mg/liter of culture medium), washed three times with 0.15 M NaCl, resuspended in their culture medium, and put back in culture for various lengths of time. In cells fractionated at the end of the first culture period, exogenous RNase was recovered mostly with the nuclear and microsomal fractions, (23% and 34%, respectively). Later, the distribution pattern shifted to one resembling that of lysosomal marker enzymes (Fig. 3). This pattern was observed up to 15 hr after resuspension with, however, some increase in soluble activity. After 20 hr of culture in the presence of RNase dimer, cell lysis occurred and most of the cell protein was recovered in the high-speed supernatant.

These results indicate that hepatoma cells handle exogenous ribonuclease dimer in the same way as normal liver cells handle RNase monomer (2), taking it up by endocytosis and storing it in their lysosomes, where it may be slowly degraded.

Labilization of lysosomes by ribonuclease dimer

In Fig. 4 are shown the results of an experiment in which a homogenate of HTC cells was incubated at 37° in a mixture of 0.25 M sucrose and 0.01 M Na–K phosphate buffer, pH 7.4, in the presence and absence of RNase dimer. Disruption of lysosomes in this preparation was followed by measurement of free *N*-acetyl-β-glucosaminidase activity. The dimer exerts a clear labilizing effect, which becomes particularly marked after 3 hr.

DISCUSSION

The results described in this paper lead to the following tentative conclusions.

(*1*) Cytotoxicity of different ribonuclease preparations on different cell types is largely a function of their rate of intracellular uptake.

(*2*) Certain tumor cells, but not rat or human fibroblasts, have high affinity binding sites for ribonuclease dimer on their surface, causing them to bind and endocytosize 10 or more times as much dimer as monomeric ribonuclease A. The selective toxicity of the dimer on tumor cells seems to be dependent on this property.

(*3*) Attachment, intracellular uptake, and cytotoxicity of ribonuclease preparations are independent of their enzymic activity. A dimer inactivated to 94% by alkylation did not differ significantly from the intact dimer in any of these properties.

(*4*) Intracellular uptake, and not simply binding to the cell surface, seems to be required for the cytostatic activity of ribonuclease preparations. Cells exposed to cytotoxic doses of such preparations do not divide but continue synthesizing DNA at a normal rate, until cytolysis sets in rather abruptly after 15–20 hr.

(*5*) Cytotoxic RNase preparations seem to accumulate intracellularly within lysosomes, as do most endocytosized materials. How they exert their cytotoxic effect is not clear. The possibility that they may cause the lysosomes to rupture is suggested by the observation that ribonuclease dimer induces lysosome labilization "*in vitro*".

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