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Antitumor Activity and Other Biological Actions of Oligomers of Ribonuclease A*

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Dimers, trimers, and tetramers of bovine ribonuclease A, obtained by lyophilization of the enzyme from 40% acetic acid solutions, were purified and isolated by cation exchange chromatography. The two conformers constituting each aggregated species were assayed for their antitumor, aspermatogenic, or embryotoxic activities in comparison with monomeric RNase A and bovine seminal RNase, which is dimeric in nature. The antitumor action was tested *in vitro* on ML-2 (human myeloid leukemia) and HL-60 (human myeloid cell line) cells and *in vivo* on the growth of human non-pigmented melanoma (line UB900518) transplanted subcutaneously in nude mice. RNase A oligomers display a definite antitumor activity that increases as a function of the size of the oligomers. On ML-2 and HL-60 cells, dimers and trimers generally show a lower activity than bovine seminal RNase; the activity of tetramers, instead, is similar to or higher than that of the seminal enzyme. The growth of human melanoma in nude mice is inhibited by RNase A oligomers in the order dimers < trimers < tetramers. The action of the two tetramers is very strong, blocking almost completely the growth of melanoma. RNase A dimers, trimers, and tetramers display aspermatogenic effects similar to those of bovine seminal RNase, but, contrarily, they do not show any embryotoxic activity.

Bovine ribonuclease A oligomerizes in the forms of dimers (1), trimers, tetramers, and higher order oligomers (2) during lyophilization from 40% acetic acid solutions. Each oligomer consists of two conformational isomers, which can be separated by cation exchange chromatography into a less basic and a more basic species (2, 3). The molecular structures of the two dimers have been solved (4, 5). They form by a three-dimensional domain-swapping mechanism (6); the less basic dimer, formerly named minor because of its ratio of 1:4 to the more basic dimer (2, 3, 5), is formed by the swapping of the N-terminal α-helix (residues 1–15) of each monomeric subunit, and the more basic or major dimer (2, 3, 5) is formed by the swapping of the C-terminal β-strand (residues 116–124) of each monomer. On this basis, the two dimers will be called N-dimer and C-dimer, respectively. The structure of the more basic or minor trimer (2, 3) has also been solved; it is formed by three monomers linked to each other by swapping their C-terminal β-strands, thereby forming a circular structure that looks like a propeller (7). It will be called the C-trimer in this paper. On the basis of its dissociation products (3, 7), a plausible linear model was proposed for the less basic, major trimer (its abundance is 1.5 times that of the more basic, minor trimer). In this linear model, two monomers are linked through swapping of their N termini, and a third monomer is bound to one of them by C-terminal domain swapping (5, 7). It will be called the NC-trimer. Two linear structures for the two tetramers, the less basic minor and the more basic major (ratio, 1:1.6), have also been proposed on the basis of their dissociation products (3, 8). The first could consist of a central dimer formed by C-terminal swapping, each monomer of which is linked to another monomer by N-terminal swapping. The second model could have the opposite structure, *i.e.* a central dimer joined by swapped N termini connected to two more monomers by the C-terminal domain-swapping mechanism. Here, they will be called the NCN-tetramer and the CNC-tetramer, respectively. These models fit the ion exchange chromatographic behavior of the two tetramers. Moreover, two additional different structures have been proposed for the RNase A tetramers by Liu and Eisenberg (8).

All RNase A oligomers, having composite active sites (His-12, His-119, and Lys-41), are enzymatically active (1, 2, 7, 9). Their specific activities show a 30–50% reduction on yeast RNA or poly(C) substrates compared with monomeric RNase A (2, 9). Although native RNase A is inactive on double-stranded RNA (dsRNA)[1] substrates under standard assay conditions (10), its oligomers show a remarkable depolymerizing activity on dsRNA. The extent of degradation of this RNA species increases in going from dimers to pentamers, and between each pair of same-sized oligomers, the ability to attack dsRNA is always higher for the more basic conformer (2, 7, 9). This activity is interpreted as being due to an initial destabilization of the nucleic acid secondary structure induced by the positive charges of the RNase molecule, *i.e.* transient single-stranded RNA stretches would form that become susceptible to ribonuclease attack (10). A correlation has indeed been found between the number of positive charges present in the active site region of mammalian pancreatic type ribonucleases and their ability to degrade dsRNA, as well as between the basic charge "den-

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[1] The abbreviations used are: dsRNA, double-stranded RNA; BS-RNase, bovine seminal RNase; cRI, cytosolic ribonuclease inhibitor.

sity" of the RNase A oligomers and their activity toward dsRNA (2, 7, 9, 10). Recently, it was also shown that the efficiency of dsRNA degradation by the RNase A dimers increases under the following conditions: (i) as the distance between the active sites of the dimer decreases (which has the effect of increasing the positive charges density at the active site region); and (ii) as the orientation of the two RNA-binding patches of the oligomeric enzyme is more twisted around the molecules (7). These parameters also hold for the C-trimer and the bovine seminal RNase (BS-RNase) (7), a dimeric basic protein (pI, 10.3) having 83% sequence identity with RNase A (11, 12, 13), whose action toward dsRNAs is quite remarkable (10, 14).

BS-RNase is also endowed with several biological actions; its aspermatogenic, embryotoxic, immunosuppressive (15–18), and, in particular, antitumor (13, 18–21) activities have been extensively studied over the years. A strong antitumor action is also exerted by onconase, a ribonuclease purified from *Rana pipiens* oocytes (22).

The lack of any significant biological activity in monomeric RNase A, and, on the contrary, the presence of various, remarkable biological actions in dimeric BS-RNase could reasonably be, at least partly, ascribed to the different quaternary structures of the two protein molecules. In fact, whereas the cytosolic ribonuclease inhibitor (cRI) (23–25) can block monomeric RNase A after its entrance into the cell, BS-RNase, because of its dimeric structure, would escape interaction with the inhibitor and therefore be able to exert its enzymatic activity in the cell (26, 27).

Based on these facts and taking into account that a significant activity against transformed cells was also shown to be displayed *in vitro* and *in vivo* by RNase A dimerized by protein engineering (28), covalently cross-linked dimers and trimers of RNase A (29–31), and a dimeric mutant of human pancreatic RNase (32), the question arose as to whether the various RNase A oligomers obtained by the lyophilization procedure and purified as described (2) might also be endowed with similar biological actions. We have therefore performed a series of *in vitro* and *in vivo* experiments, which demonstrate that RNase A dimers, trimers, and tetramers display aspermatogenic and antitumor activities that increase remarkably as a function of the oligomer mass and, at the same time, show a complete lack of embryotoxicity.

EXPERIMENTAL PROCEDURES

Preparation of RNase A Oligomers—Dimers, trimers, and tetramers of RNase A (Type XII-A, purchased from Sigma) were prepared by lyophilization of the protein from 40% acetic acid solutions, as described (2). The lyophilized material, dissolved in 0.08 M sodium phosphate buffer, pH 6.7, was subjected to ion-exchange chromatography with a Source 15S HR 16/10 or 16/50 column in a fast paced liquid chromatography (FPLC) system (Amersham Biosciences). Separation of the various RNase A species, at room temperature, was performed at pH 6.7 using a 0.085–0.18 M sodium phosphate gradient with the 16/10 column (2) or a 0.09–0.20 M sodium phosphate gradient with the 16/50 column. The aggregates, if not used immediately, were kept diluted and frozen until use. In fact, the stability of the RNase A aggregates in sodium phosphate buffer, pH 6.7, is definitely higher at low protein concentration (3). The concentration of RNase A was estimated spectrophotometrically using $\epsilon_{280}^{1\%} = 7.3$ (33).

Bovine Seminal RNase—Bovine seminal ribonuclease was isolated from bull seminal plasma obtained from healthy, sexually mature bulls bred in the Litohor insemination station (Litohor, Czech Republic). To isolate the enzyme, one volume of the seminal plasma was diluted with 2.5 volumes of 2% acetic acid. The protein precipitate was removed by centrifugation, and solid ammonium sulfate was added to the supernatant up to 3 M concentration. After another centrifugation, the new supernatant was then dialyzed using the Vivaflow 50 linked module flow system (Vivascience Ltd., Stonehouse, UK) and freeze-dried. Chromatographies with CM Sephadex C-50 and Sephadex G-100 columns, respectively, were used for complete purification (34).

Cathodic Gel Electrophoresis—Cathodic gel electrophoresis under nondenaturing conditions was performed according to Goldenberg (35) with slight modifications using a β-alanine/acetic acid buffer, pH 4. Gels (12% polyacrylamide) were run at 20 mA for about 100 min at 4 °C. Fixing and staining were performed with 12.5% trichloroacetic acid and 0.1% Coomassie Brilliant Blue.

Aspermatogenic Effects—Assays for the aspermatogenic action of BS-RNase or the various RNase A oligomers were carried out as described elsewhere (34). A 0.01–0.05-ml volume of each sample containing 100 μg of enzyme protein was injected into the left testis of five ICR mice. After 10 days, the left and right (control) testes were excised and studied histologically. Degenerative effects were assessed, namely decreased weight of the testes, decreased width of spermatogenic layers, and reduced seminiferous tubules diameter.

In Vitro Assays for Antitumor Activity—The antitumor action of the various RNase A aggregates was assayed in comparison with that of BS-RNase using two human tumor cell lines, ML-2 and HL-60, originally derived from patients suffering from acute myeloid leukemia. The cells were cultured in microtiter plates containing RPMI 1640 medium supplemented with 10% fetal calf serum exposed to a humidified atmosphere enriched with CO_2 (5%, v/v). After the addition of the different enzyme preparations, the cells were cultured for 48 h. Four hours before ending the experiment, cells were pulse-labeled with 24 kBq of [6-^{3}H]-thymidine (specific activity, 980 GBq/mmol, Institute for Research, Development and Application of Radioisotopes, Prague, Czech Republic). The biological action was expressed as counts per min (cpm), and inhibition of DNA synthesis was expressed as a percentage of controls. These experiments were carried out in triplicate.

In Vivo Assays for Antitumor Activity—The antitumor action was tested in CD-1 athymic strain female outbred nude mice (AnLab Ltd., Charles River Laboratories, Prague, Czech Republic) weighing between 18 and 20 g. The mice (42, divided into seven groups of six mice each) were kept under aseptic conditions in cages with bedding (SAWI-Research bedding, AnLab Ltd.) sterilized by irradiation. They were fed with a radiation-sterilized ST-1 (Bergman) diet and given autoclaved water *ad libitum*. Human non-pigmented melanoma (line UB900518), obtained from a surgical specimen cut in small pieces (3 × 3 mm), was transformed (stabilized) in tissue culture. This material was transplanted subcutaneously (1×10^7 cells/nude mouse, with 0.1 ml of Matrigel) into the right flank of the nude mice. Treatment was started when the area of the transplanted tumor reached the size of 5 × 5 mm (0.15–0.26 cm^3), which occurred ~14 days after inoculation. The RNase A oligomers were administered intravenously in doses of 250 μg/20 g three times a week for 4 weeks. Saline solution was administered to control animals (a group of 11 mice). Tumor sizes were measured twice a week using a slide caliper, and volume was calculated as $V = a \times b \times \pi/6$, where a and b are the long and short dimensions, respectively. The percentage of tumor growth inhibition (1 − (mean tumor volume in treated group/mean tumor volume in controls) × 100) was calculated and shown in Fig. 3.

Embryotoxic Effects—Two-cell embryos were obtained from superovulated mice by flushing mouse oviducts ~36 h after mating. Embryos were cultured in CZB medium (81.62 mM NaCl, 4.83 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$-$7H_2O$, 25.12 mM $NaHCO_3$, 1.7 mM $CaCl_2$-H_2O, 31.3 mM sodium lactate, 0.27 mM sodium pyruvate, 0.11 mM EDTA (disodium salt), 1 mM glutamine, 100 units/ml penicillin G sodium, 0.50 mg/ml streptomycin, and 3 mg/ml bovine serum albumin) and the protein species to be tested (μg/ml; see Table II) for 72–96 h in a humidified atmosphere containing CO_2 (5% v/v) at 37.5 °C (36). Controls were prepared as above, but without the protein species to be tested. The developmental stage of the embryos was monitored with a stereomicroscope.

RESULTS

Aspermatogenic Activity of the RNase A Oligomers—Table I shows the results of the injection of 100 μg of each of the two conformational isomers of dimeric, trimeric, or tetrameric RNase A into the left testis of a series of mice. The parameters studied to establish the degree of the aspermatogenic effect were the weight of the testes, the width of the spermatogenic layers, and the diameter of the seminiferous tubules of testes. The action of the oligomers was compared with those of native RNase A and BS-RNase. Although the statistical significance of the data might be modest because of the small number of samples, it is sufficiently clear that, although monomeric RNase A was devoid of any effect, the activities of the RNase A oligomers were, in general, similar to those displayed by BS-

TABLE I

Aspermatogenic action of RNaseA oligomers compared with that of RNase A or BS-RNase

100 μg of each protein species were injected into the left testis of ICR mice. After 10 days, the left and right (control) testes were excised and studied histologically. Aspermatogenic effects were assessed as the decreased weight of testes, the reduced width of spermatogenic layers, and the reduced diameter of seminiferous tubules. PBS, phosphate-buffered saline; N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C-trimer; NCN_{TT}, NCN-tetramer; CNC_{TT}, CNC tetramer.

RNase A aggregates injected	Number of mice	Index weight of testes ± S.D.		Width of spermatogenic layers of testes ± S.D.		Diameter of seminiferous tubules of testes ± S.D.		Degree of Aspermatogensis
		Injected testes	Non-injected testes	Injected testes	Non-injected testes	Injected testes	Non-injected testes	
				μm		μm		
PBS (control)	6	41 ± 9	42 ± 7	61 ± 8	58 ± 10	152 ± 7	153 ± 13	0
RNase A	5	42 ± 4	43 ± 2	64 ± 3	62 ± 6	150 ± 9	156 ± 6	0
BS-RNase	6	19 ± 8	32 ± 10	36 ± 3	62 ± 8	139 ± 13	165 ± 11	2–3
N_D	5	40 ± 3	43 ± 3	30 ± 5	60 ± 5	151 ± 11	153 ± 14	2–3
C_D	5	39 ± 3	40 ± 1	37 ± 7	59 ± 11	151 ± 5	155 ± 6	2–3
NC_T	5	35 ± 3	36 ± 3	36 ± 4	63 ± 6	144 ± 6	157 ± 9	2–3
C_T	5	36 ± 6	38 ± 1	28 ± 11	63 ± 4	143 ± 18	156 ± 8	2–3
NCN_{TT}	5	50 ± 18	42 ± 2	54 ± 12	60 ± 4	157 ± 21	155 ± 5	1–3
CNC_{TT}	5	40 ± 3	40 ± 1	41 ± 15	61 ± 7	151 ± 22	161 ± 7	2–3

RNase with the exception of the index weight of testes, which was not significantly reduced by the RNase A oligomers. Moreover, the more basic tetramer (CNC), appears to have a slightly higher aspermatogenic activity than the less basic one (NCN).

Embryotoxic Effects of the Various Aggregates of RNase A—The development of mice embryos after 72 h incubation with the two dimers or trimers of RNase A (100 μg/ml) and a mixture of the two tetrameric conformers is shown in Table II. The action of RNase A oligomers has been tested in parallel with that of BS-RNase and native RNase A. It is quite clear that, whereas 50% of two-celled-embryos reached the blastocyst stage in control experiments and 44% in the presence of monomeric RNase A, only 10% of the embryos grew to blastocysts in the presence of BS-RNase. On the contrary, no embryotoxicity was displayed by any of the aggregated species of RNase A, in the presence of which 67–82% of the embryos formed blastocysts.

In Vitro Antitumor Activity of the Oligomers of RNase A—The action of the various RNase A oligomers on ML-2 (human myeloid leukemia) cells is shown in Figs. 1, *A* and *B* and 2, *A* and *B*. The activities of the two dimeric and trimeric conformers, compared with those of BS-RNase, are shown in Fig. 1*A*. The two dimers and the two trimers display lower antiproliferative activities than BS-RNase. Although the N-dimer is definitely less active than the C-dimer, the two trimers show similar activities. Moreover, the C-dimer and the NC-trimer, whose charge characteristics are similar (they elute quite close to each other off cation exchange chromatography) (2), also show similar antiproliferative activities. The results obtained with the two tetramers are shown in Fig. 1*B*. Whereas the antiproliferative action of the NCN-tetramer is similar to or slightly lower than that of BS-RNase, the CNC-tetramer, the more basic of the two, is definitely more active than both the NCN-tetramer and BS-RNase.

Qualitatively similar results were obtained by testing the antiproliferative action of RNase A dimers, trimers, or tetramers in comparison with that of BS-RNase on HL-60 cells, a human myeloid cell line. Fig. 2*A* shows the action of the two dimeric and trimeric conformers. With a dose of 10 μg/ml, the two dimers and the two trimers appear to display similar activities, but they are lower than the activities of BS-RNase. At doses higher than 20 μg/ml for the N-dimer and than 10 μg/ml for the other RNase A oligomers, their antiproliferative action is certainly higher than that of BS-RNase. As for the two tetramers (Fig. 2*B*), they are undoubtedly the most efficient antiproliferative agents; the CNC-tetramer, the more basic of the two, appears to be more active than the NCN-tetramer.

In Vivo Antitumor Activity of the Oligomers of RNase A—The results of the last of three different series of experiments, which are qualitatively very similar to each other, are presented in Fig. 3. It is quite clear that all of the RNase A oligomers are active against the growth of human melanoma in nude mice with their action increasing as a function of the size of the oligomers, which is in agreement with the results obtained in the *in vitro* experiments performed on the ML-2 or HL-60 cell lines. The inhibition of tumor development exerted by trimers and tetramers is particularly strong, with the latter definitely being the most efficient antitumor agents. The only discrepancy concerns the reciprocal extent of action displayed by the two dimeric conformers; the more basic dimer (C-dimer) shows less activity than the less basic (N-dimer), whereas in the *in vitro* experiments the opposite result was found. It might also be worth mentioning that no significant changes in body weight of the six series of mice treated with dimers, trimers, or tetramers of RNase A could be noticed in the course of the experiment (data not shown).

DISCUSSION

The results reported in this work show that the oligomers of bovine ribonuclease A, obtained by lyophilizing the enzyme protein from 40% acetic acid solutions, are endowed with some biological actions. As described in the Introduction, the structures of the two dimers and one of the two trimers have been determined, whereas for the second trimer and the two tetramers plausible models have been proposed on the basis of their dissociation products (3–5, 7). The biological actions of the oligomers consist in an *in vitro* and *in vivo* antitumor activity (Figs. 1, 2, and 3), as well as an aspermatogenic action (Table I), similar to those ascertained for bovine seminal RNase. The RNase A oligomers lack, instead, the embryotoxic activity, which accompanies the cytotoxic action of BS-RNase (Table II).

Several points need to be discussed. First, how can RNase A oligomers enter the cells? We do not have any direct evidence about this, but we might assume that they could bind to the cell surface by adsorption and then enter the cell by endocytosis, as has been suggested for BS-RNase (26, 37, 38) and some RNase A variants endowed with potent cytotoxic activity (32, 38, 39). In this connection, the importance of the polyanionic nature of the surface of mammalian cells for their interaction with cationic proteins has been mentioned by Kim *et al.* (26), and RNase A oligomers are definitely cationic molecules. In keeping with this model, RNase Sa, a negatively charged RNase (pI, 3.5), does not show cytotoxicity, but a variant with five carboxylate to lysine substitutions (pI, 10.2) does (40). Moreover, when the highly positively charged N-terminal β-hairpin of α-sarcin is removed by site-directed mutagenesis, its cytotoxic effects are

TABLE II

Development of mice embryos after 72 hours incubation with RNase A oligomers, RNase A, or BS-RNase

Two-cell embryos were obtained from superovulated mice by flushing mouse oviducts 36 h after mating. Embryos were cultured in CZB medium supplemented with bovine serum albumin (3 mg/ml) and the protein to be tested for 72–96 h at 37.5 °C (CO_2, 5% v/v). The developmental stage of embryos was monitored with a stereomicroscope. Quantity of each enzyme species was 100 μg/ml. N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C-trimer; NCN_{TT}, NCN-tetramer; CNC_{TT}, CNC tetramer.

Enzyme species added to the medium used for embryo culture	Number of mice embryos	Number of embryos in cell stages			
		Blastocysts	Expanded blastocysts	Total blastocysts	Blastocysts
					%
Control (medium)	10	1	4	5	50
RNase A	9	1	3	4	44
BS-RNase	10	1	0	1	10
N_D	11	5	4	9	81
C_D	17	10	2	12	70
NC_T	11	5	4	9	82
C_T	12	6	2	8	67
NCN_{TT} + CNC_{TT}	19	13	2	15	79

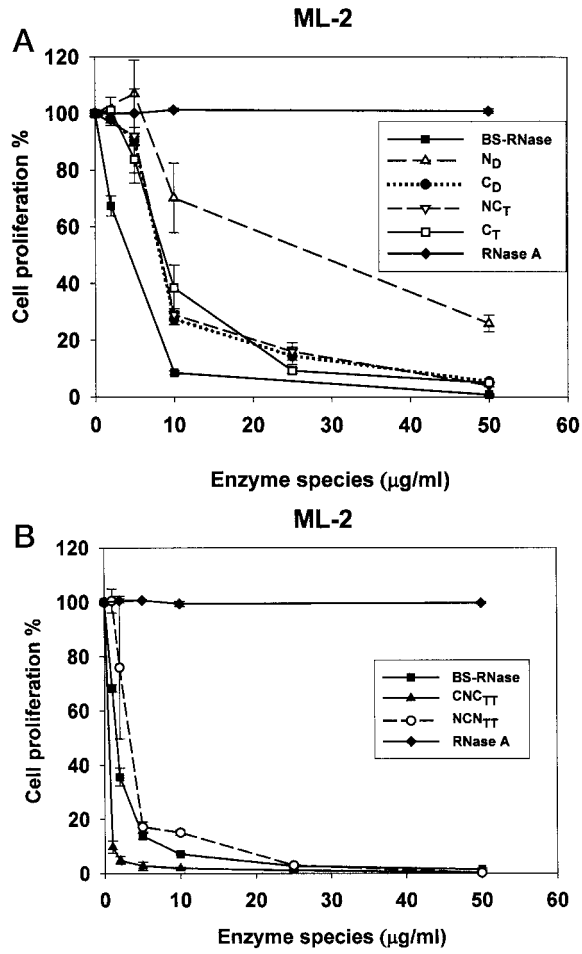

FIG. 1. **Action of RNase A oligomers and BS-RNase on the proliferation of ML-2 cells.** *A*, action of RNase A dimers and trimers and BS-RNase on the proliferation of ML-2 cells. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in CO_2 atmosphere (5%, v/v). After addition of the various enzyme species, culturing of the cells was protracted for 48 h. Four hours before ending the experiment, cells were pulse-chased with [^{3}H]thymidine (see "Experimental Procedures"). Activity is expressed as counts per minute, and inhibition of DNA synthesis is expressed as a percentage of control. Experiments were performed in triplicate. N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C trimer. *B*, action of tetramers of RNase A and BS-RNase on the proliferation of ML-2 cells. Experiments (in triplicate) were carried out as described above for *A*. CNC_{TT}, CNC-tetramer; NCN_{TT}, NCN tetramer.

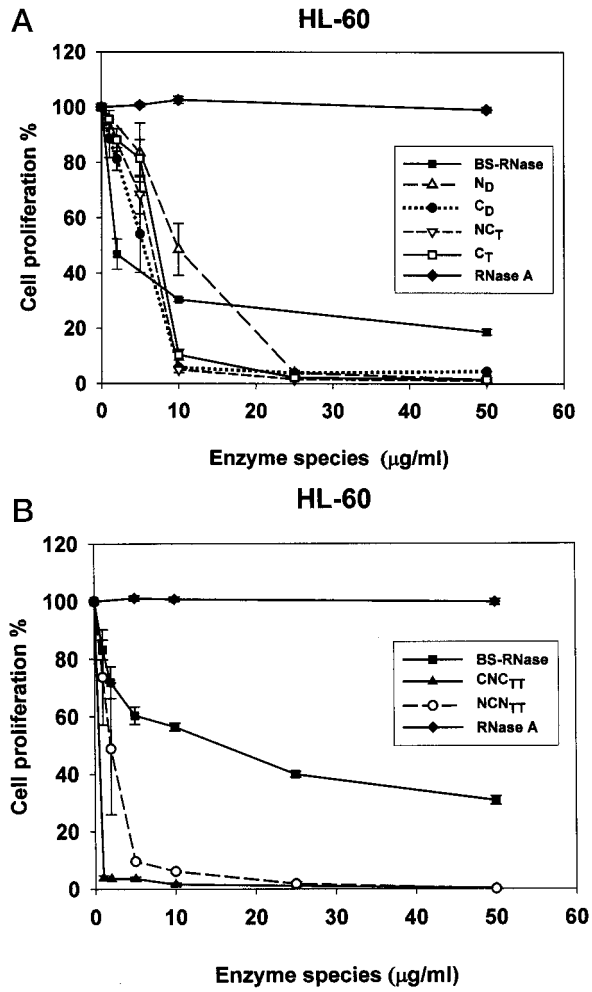

FIG. 2. **Action of RNase A oligomers and BS-RNase on the proliferation of HL-60 cells.** *A*, action of dimers and trimers of RNase A and the action of BS-RNase on the proliferation of HL-60 cells. Experiments (in triplicate) were carried out as described in the legend to Fig. 1*A*. N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C trimer. *B*, action of RNase A tetramers and BS-RNase on the proliferation of HL-60 cells. Experiments (in triplicate) were carried out as described in the legend to Fig. 1*A*. CNC_{TT}, CNC-tetramer; NCN_{TT}, NCN tetramer.

greatly reduced (41). These facts point out the importance of the presence of positive charges on the ribonuclease molecule for its entrance into the cell and its cytotoxic activity. Although the endosomes and the trans-Golgi network have been identified as essential steps on the route of BS-RNase from the extracellular matrix to the cytosol of malignant cells (42), we have no indication of the pathway that dimers, trimers, and tetramers of RNase A follow after entering tumor cells. Once the RNase A oligomers are in the cytosol, they may display

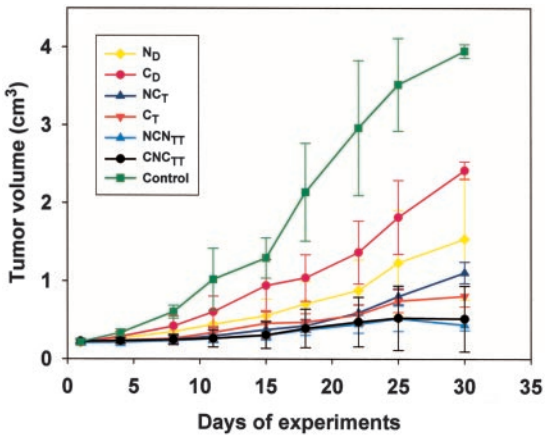

FIG. 3. **Inhibition by the action of dimers, trimers and tetramers of RNase A of the growth of human melanoma transplanted in nude mice.** Human non-pigmented melanoma (line UB900518) was stabilized in tissue culture and transplanted subcutaneously in six series of six nude mice each (plus one series of controls). Each inhibition value shown is the mean of six measurements. Details are given under "Experimental Procedures." The RNase A oligomers were administered intravenously with a dose of 250 μg/20 g three times a week for four weeks. Saline solution was administered to control animals. The action of the various RNase A species was calculated as described under "Experimental Procedures." N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C trimer; CNC_{TT}, CNC-tetramer; NCN_{TT}, NCN tetramer.

their enzymatic action against the various cellular RNA species. This would, in principle, also occur with monomeric RNase A if the enzyme was not totally inhibited by its interaction with the cytosolic inhibitor, cRI (23–25), which binds RNase A with a K_d of 10^{-15} M^{-1} (43). The structure of cRI (44), although fitting a monomeric ribonuclease, should be unable to interact efficiently with a dimeric RNase, as shown by the inability of cRI to inhibit not only the action of the naturally dimeric bovine seminal ribonuclease (26) but also that of RNase A dimerized by protein engineering (28) as well as the action of the dimeric variant of human pancreatic RNase (32). The importance of the structure of the ribonuclease in cRI complex (45) is also supported by the observation that bovine RNase A, if conjugated to polyethylene glycol (PEG), acquires aspermatogenic and antitumor activities similar to those of BS-RNase (46), possibly because the conjugated PEG sterically hinders the interaction with cRI. Similarly, the cytotoxic activity of the various RNase A oligomers tested by us could be ascribed to their ability to escape the interaction with cRI because of their size. However, cytotoxicity is not necessarily linked to the dimeric or oligomeric nature of a ribonuclease molecule. In fact, onconase and angiogenin are both monomeric and cytotoxic, but both proteins need their ribonucleolytic activity to display their biological actions. In the case of the RNase A oligomers, one point deserves consideration, *i.e.* the antitumor action of dimers, trimers, and tetramers increases with the size of the oligomer, with the action becoming very strong in the case of the two tetramers. On the one hand, this can be attributed to the increasing ability of the oligomer, in going from dimers to tetramers, to escape interaction with the cRI because of its increasing size. On the other hand, this picture could be rather naive, because in principle there should not be any great difference in the escaping ability of an RNase A dimer *versus* an RNase A tetramer. Rather, the parallelism between the action of oligomeric RNase A against tumor cells (increasing with the size of the oligomers) and the catalytic activity of the oligomers against double-stranded RNA, which also depends on their masses (in terms of the positive charge density on the oligomeric molecules, which also increases with their size) (2, 7, 9), should not escape our attention. In other words, both properties could be linked to each other. Moreover, we must point out that, within each pair of same-sized oligomers, the more basic conformer is generally more active than the less basic conformer both against tumor cells and at degrading dsRNA (2, 7, 9), the only discrepancy being in regard to the *in vivo* antitumor activity of the two dimers (Fig. 3). In conclusion, the degree of basicity of an RNase molecule might be the root of three important events, *i.e.* its entrance into the cells (26), its ability to degrade dsRNA, and its antitumor activity. In this regard, the relatively low antitumor action of covalently linked RNase A oligomers (29–31), as well as their lower degrading activity toward double-stranded RNA compared with that of the corresponding aggregated dimers and trimers (31), could be ascribed to the loss of some positive charges due to the involvement of lysine residues in the cross-linking reaction performed with dimethyl suberimidate (10). With regard to the selective antitumor action of RNase A oligomers observed *in vivo*, it must also be recalled here that the dimers and higher oligomers of RNase A degrade DNA:RNA hybrids (47) as well as dsRNA, as does BS-RNase (48). Whereas it is unlikely that an RNase could enter the nucleus of a normal cell, the regulation of a cancer cell over the entry of proteins through the nuclear pore might be definitely lower, letting the RNase inside. Moreover, the nuclear membrane breaks down during cell division, and cancer cells divide much more frequently than normal cells. Therefore, although all double-stranded regions of cellular RNAs, in particular those present in tRNA, can be more effectively degraded by oligomeric ribonuclease A, DNA:RNA hybrid stretches, like those occurring in the priming of DNA synthesis by RNA or the synthesis of mRNA, could also be efficient substrates for the RNase A oligomers in their uncontrolled action inside the cells. Another point to be considered in relation to the biological activities reported here is the survival of the various RNase A oligomers in solution and in *in vitro* or *in vivo* experiments. The stability of dimers, trimers and tetramers, dissolved in different buffers at neutral pH, was studied as a function of temperature, and the kinetics of the dissociation of trimers and tetramers at 35 °C was measured (3, 49). The results consistently indicated that dimers are relatively more stable than trimers and tetramers, the latter being the least stable oligomers. However, it is rather difficult to envisage the stability of each type of aggregate under quite different conditions, *i.e.* when they are added to cell cultures or injected into neoplastic and/or normal tissues. The relative stabilities of dimers, trimers, and tetramers should not change, but their absolute stability values could be highly modified under *in vitro* or *in vivo* experimental conditions. For example, the aspermatogenic activity of the RNase A oligomers shown in Table I could have been reduced by the possible partial dissociation of the oligomers, in particular the two tetramers, during the 10 days of treatment. It might also be worth considering here that, based on the higher stability of the C-dimer over the N-dimer (49), the hypothesis could be advanced that the higher antitumor activity generally observed in this work for the C-terminal swapped dimer, the C-trimer, or the CNC-tetramer might not only be related to their higher basicity but also to their more stable structures in relation to the ability to evade cRI. For instance, the CNC-tetramer comprises two C-terminal swapped dimers, whereas two N-terminal swapped dimers are present in the NCN-tetramer. Accordingly, the latter shows *in vitro* a lower antitumor activity than that displayed by the CNC-tetramer. However, it has to be taken into account that, although, as mentioned above, under all experimental conditions tested the stability of the RNase A oligomers undoubtedly decreases in going from dimers to tetramers, for each oligomeric species the stability of one of the two conformers relative

to that of the other appears to be greatly influenced by the environmental conditions (3, 49).[2] In conclusion, however, the activity patterns shown in Figs. 1, 2, and 3 indicate that each oligomeric species should survive long enough to allow the effects described. Moreover, the dissociation of the larger oligomers (trimers and tetramers) does not necessarily result in complete inactivity, because aggregates of smaller size are transiently produced (3, 7). Furthermore, the singular lack of embryotoxicity shown by RNase A oligomers could indeed also be related to the relative instability of the various oligomers. Although it is difficult to envisage to what the embryotoxic action of BS-RNase may be ascribed and how it develops, it might be reasonable to think that the innocuity of the various RNase A oligomers could be related to their relative instability and short survival time under the conditions of both *in vitro* and *in vivo* experiments.

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Antitumor Activity and Other Biological Actions of Oligomers of Ribonuclease A

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