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Comprehensive comparison of the cytotoxic activities of onconase and bovine seminal ribonuclease

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Abstract

Onconase (ONC) and bovine seminal ribonuclease (BS-RNase) are homologs of bovine pancreatic ribonuclease (RNase A). Unlike RNase A, ONC and BS-RNase can evade the cytosolic ribonuclease inhibitor protein and are potent cytotoxins. Here, the endogenous cytotoxic activities of ONC and BS-RNase are compared in a wide variety of assays. Injections of ONC into one or both testes of mice and rats evokes a stronger aspermatogenic activity than does the injection of BS-RNase. Epididymides exposed to ONC lose mass and all sperm. Testicular tissue is gradually colonized by immunite and fibrocytic cells. Yet, Leydig cells are always present and functional in the ligamented parts of testicles injected with ONC or BS-RNase. ONC is likewise more toxic to mouse embryos than is BS-RNase, both in vitro and in vivo. The antiproliferative effect of ONC on human tumor cell line ML-2 and lymphocytes in a mixed lymphocyte culture is also more pronounced than is that of BS-RNase. The number of granulocyte-macrophage colony-forming units is repressed almost completely by ONC, whereas a five-fold higher dose of BS-RNase does not cause substantial inhibition. In mice, ONC is less immunogenic than BS-RNase but more immunogenic than RNase A. Together, these data indicate that ONC is a pluripotent cytotoxin, and serves as the benchmark with which to gauge the cytotoxicity of other ribonucleases.
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Keywords: Aspermatogenesis; Cancer; Chemotherapy; Embryotoxicity; Epididymides; Immunogenicity; Leydig cells; Ribonuclease A; Ribonuclease inhibitor; Testes

1. Introduction

Ribonucleases can be cytotoxic because cleavage of RNA renders indecipherable its encoded information. The cytotoxicity of pancreatic-type ribonucleases was discovered in the 1950s. At that time, bovine pancreatic ribonuclease (RNase A; EC 3.1.27.5; D'Alessio and Riordan, 1997; Raines, 1998) was shown to be toxic to tumor cells, both in vitro (Ledoux and Baltus, 1954) and in vivo (Ledoux, 1955a,b; Alexsandrowicz, 1958). Although effects in vivo were observed only after milligrams of enzyme were injected into solid

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tumors, these early studies were the first to demonstrate the potential of pancreatic-type ribonucleases as chemotherapeutics.

More recently, two homologs of RNase A were found to have much greater endogenous cytotoxic activity than RNase A itself. In the early 1970s, bovine seminal ribonuclease (BS-RNase; Fig. 1) was discovered and shown to be a potent cytotoxin (Hosokawa and Irie, 1971; D'Alessio et al., 1972; Dostál and Matousek, 1972). The degenerative effect of BS-RNase on spermatogenic epithelium in mice (Dostál and Matousek, 1973; Leone et al., 1973) and other animal species (Matousek, 1974) was demonstrated soon thereafter. In the late 1980s, Onconase® (ONC; Fig. 1) was isolated from the oocytes and early embryos of the Northern leopard frog, *Rana pipiens* (Darzynkiewicz et al., 1988; Ardelt et al., 1991). This cytotoxin has advanced to Phase III clinical trials (USA) as a cancer chemotherapeutic (Mikulski et al., 2002).

The mechanism by which ONC and BS-RNase kill cells is under much scrutiny (for recent reviews, see: D'Alessio et al., 1997; Youle and D'Alessio, 1997; Leland and Raines, 2001; Matousek, 2001; Makarov and Ilinskaya, 2003). It is known that their ribonucleolytic activity is essential for their cytotoxicity (Newton et al., 1994; Kim et al., 1995b). This ribonucleolytic activity can be manifested in cells because ONC and BS-RNase (Murthy and Sirdeshmukh, 1992; Wu et al., 1993), unlike RNase A (Lee et al., 1989; Vicentini et al., 1990), can evade the ribonuclease inhibitor protein that resides in the cytosol of mammalian cells (for recent reviews, see: Hofsteenge, 1997; Shapiro, 2001).

BS-RNase is known to have broad cytotoxicity. For example, BS-RNase isolated from bull seminal plasma stops the development of mouse embryos in culture (Matousek, 1973a), and interrupts gravidity in pregnant guinea pigs, rats, and rabbits (Matousek, 1973b, 1975). The toxicity of BS-RNase for Crocker tumor cells and other cancerous cells (such as Walker carcinosarcoma and Sajdel hepatoma in rats (Stanek and Matousek, 1976), leukaemic cells in mice (Matousek and Stanek, 1977), and HeLa, leukemic, and lymphoblastoid cells of humans (Cinatl et al., 1977; Soucek and Matousek, 1979; Soucek et al., 1996a)) is consistent with embryonic and tumor cells sharing common features (Matousek, 1973b). The absence of harmful effects on human bone marrow cells treated with BS-RNase suggests a specificity against cancer cells (Soucek et al., 1986). These cytotoxicity results have been replicated with BS-RNase prepared by recombinant DNA technology (Kim et al., 1995a,b,c), and the toxicity of the recombinant enzyme has been extended to oocyte maturation (Slavík et al., 2000). Recently, ONC has likewise been prepared by recombinant DNA technology (Boix et al., 1996; Leland et al., 1998; Natomista et al., 1999; Vorobiev et al., 2001).

Here we compare the aspermatogenic, embryotoxic, antitumor, and other biological activities of recombinant ONC to those of BS-RNase and RNase A. The results indicate that ONC and BS-RNase, unlike RNase A, are potent cytotoxins to a variety of cell types. In general, however, ONC is a more potent cytotoxin than is BS-RNase. Thus, ONC is the benchmark with which to gauge the cytotoxicity of other ribonucleases.

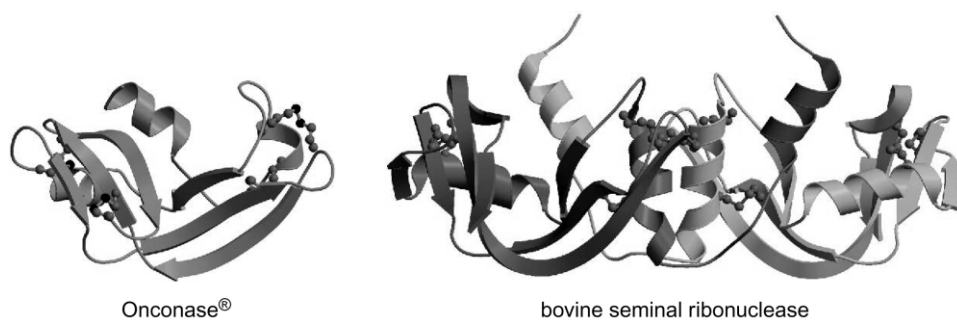Onconase®   bovine seminal ribonuclease

Fig. 1. Three-dimensional structure of ONC (PDB entry 1ONC Mosimann et al., 1994) and BS-RNase (PDB entry 1BSR Mazzarella et al., 1993) on the same scale. The atoms of cystine residues are shown explicitly. BS-RNase also exists in a second quaternary form in which the N-terminal segments are not swapped between the monomeric subunits (Berisio et al., 2003).

2. Materials and methods

2.1. Preparation of ribonucleases

RNase A was prepared from bovine pancreas and obtained from ICN Biomedicals (Zlín, Czech Republic). The enzyme was free of aggregates, and had a specific activity of 75 Kunitz units/mg.

BS-RNase was prepared from bull seminal plasma as described previously (Michaelis et al., 2002). Briefly, one volume of bull seminal plasma was diluted with 2.5 volumes of aqueous acetic acid (2% v/v). The protein precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant (to 3 M). This solution was then dialysed using Vivaflow 50 Linked Modules (Vivascience, Stonehouse, UK), and lyophilized. The material was purified further by cation-exchange chromatography on a CM Sephadex C-50 column using a linear gradient of NaCl (0.10–0.50 M) in 0.050 M sodium phosphate buffer, pH 8.0. BS-RNase eluted at a concentration of 0.36 M NaCl. This fraction was purified by gel-filtration chromatography on a Sephadex G-100 column equilibrated with 0.10 M Tris–HCl buffer, pH 7.5. The protein solution was dialysed again using Vivaflow 50 Linked Modules, and lyophilized.

ONC was produced in *Escherichia coli* by recombinant DNA technology, and purified as described previously (Leland et al., 1998; Lee and Raines, 2003).

2.2. Assay of aspermatogenic activity with one testis

Sexually adult male ICR mice were injected in their left testis with ONC, BS-RNase, or RNase A (100 μg). Ten days after the injections, the animals were sacrificed and examined. The overall aspermatogenic activity was quantitated by measuring the index mass, which is $10 \times$ testis mass (in mg)/body mass (in gram). The destructive effect on the testis was evaluated by the loss of spermatogenic cells, and the decrease in the width of spermatogenic layers and diameter of seminiferous tubules. The degree of testicular damage was assessed on a scale from 0 (normal histological appearance) to 4 (disappearance of all cells of the spermatogenic layers and the spermatogonia of the convoluted tubules). Tubules (50–60) from the central part of the testis were examined by microscopy.

2.3. Assay of aspermatogenic activity with both testes

Both testes of mice and rats were injected once with 0.10 mg and 1.0 mg, respectively, of a ribonuclease. Both testes with their epididymides and seminal vesicles were excised after 20, 40, 60, 80, or 100 days from mice, and after 100 days from rats. These sexual organs were weighed and characterized by histology, as described above. Special attention in histological observation was devoted not only to spermatogenic cells, but also to Leydig cells and the infiltration of leukocytes (mononuclear and polymorphonuclear cells) and lymphocytes passing trough the tunica albuginea. The degree of connective tissue with collagen fibres, fibroblasts, and fibrocytes was included in the histological assessment of excised testes.

Epididymides were extirpated from testes, weighed, and characterized by histology to determine the number of spermatozoa present. The number of sperms in epididymides were counted by microscopy in a special ocular with a 3-mm window and $1000 \times$ magnification. The mass of seminal vesicles and their histology were also compared in treated and normal fertile mice.

2.4. Testosterone radioimmunoassay

The concentration of testosterone in blood plasma was assayed with the use of a Testosterone Direct radioimmunoassay kit (Immunotech, Marseille, France). The cross-reactivity of the anti-testosterone antibody was as follows: testosterone, 100%; 5α-dihydrotestosterone, 10%; 11β-hydroxytestosterone, 2%; 4-androstenedione, 0.6%; 19-nortestosterone, 5%; and methyltestosterone, 2%. The cross-reactivity with other steroids was $<0.03\%$. Radioimmunoassays were performed according to the kit instructions. Briefly, standard, control, or plasma samples (50 μl) were dispensed into antibody-coated tubes. After the addition of a solution (500 μl) of ^{125}I-labelled testosterone tracer, the tubes were incubated for 3 h at 37 °C. The liquid in the tubes was then removed by aspiration, and the radioactivity in each tube was quantitated with a gamma radiation counter (Berthold Multi Gamma 2104, Berthold, Germany). The detection

limit of the assay was 0.029 ng/ml, and the coefficient of variance within an assay was 3.83%.

2.5. Assay of embryotoxic activity in vitro

Ribonucleases were assayed for an effect on the development of mouse embryos in vitro. Two-cell embryos from superovulated C57/BL6 mice were flushed from oviducts ca. 36 h after mating. Embryos were cultured in CZB medium supplemented with bovine serum albumin (3 mg/ml) and a ribonuclease (ONC, 1–100 μg/ml; BS-RNase, 100 μg/ml; or RNase A, 100 μg/ml) for 72–96 h at 37.5 °C in a humidified atmosphere containing $CO_2(g)$ (5% v/v). The developmental stage of the embryos was then evaluated by microscopy (Chatot et al., 1989).

2.6. Assay of embryotoxic activity in vivo

Ribonucleases were assayed for an effect on the development of mouse embryos in vivo. The embryos of female mice (ICR) were injected with various concentrations of ONC or BS-RNase, and the mice were sacrificed at various times after mating. (The time of mating was estimated from the discovery of copulation plugs.) Four days after the injection, mice were killed by the inhalation of ether, and the number of live and dead embryos was determined by inspection.

2.7. Assay of antitumoral activity

The antitumoral activity of ONC and BS-RNase was assayed using cell line ML-2, which was derived from a human myeloid leukaemia, as described previously (Soucek et al., 1996b). Briefly, cells (2×10^5) in RPMI 1640 medium (0.2 ml) supplemented with fetal calf serum (10% v/v) were established in microtiter plates (NUNC, FB type) and cultivated in humidified atmosphere containing $CO_2(g)$ (5% v/v) for 48 h. Simultaneously, a known concentration of ONC or BS-RNase was added to the cultures. Four hours before the termination of cultivation, samples were pulsed with 24 kBq of [6-³H]thymidine (specific activity 980 GBq/mmol, Institute for Research, Development and Application of Radioisotopes, Prague, Czech Republic). Cells were then collected with a Scatron harvester, and the incorporated radioactivity was quantitated with a Beckman scintillation counter.

2.8. Assay of immunosuppressive activity

The immunosuppressive activity of ONC and BS-RNase on human lymphocytes stimulated in mixed lymphocyte culture (MLC) was assessed as described previously (Soucek et al., 1999). Lymphocytes from two unrelated donors were mixed and cultivated in a humidified atmosphere containing $CO_2(g)$ (5% v/v) at 37 °C in RPMI 1640 medium supplemented with mixed human AB serum (10% v/v). A known concentration of ONC or BS-RNase was added at the beginning of experiment, and the cell mixture was incubated for 6 days. The pulsation with [6-³H]thymidine was then carried out as described above.

2.9. Assay of colony-forming activity with bone marrow cells

The effect of ONC and BS-RNase on human bone marrow cells was assessed by using a colony-forming unit-granulocyte-macrophage (CFU-GM) assay as described previously (Matousek et al., 2001). Human bone marrow cells were obtained by sternal puncture of a normal donor. The cells were collected in Iscove medium mixed 1:1 with ACD, supplemented with fetal calf serum (to 20% v/v) and conditioned medium 5637 (to 10% v/v), and cultivated in semisolid agar medium as described previously (Metcalf, 1977). Bone marrow cells (1×10^6) were preincubated for 1 h with various concentrations of ONC or BS-RNase in complete cultivation medium (5 ml). An aliquot (4.5 ml) of this suspension was mixed with agar (0.5 ml of a 3.3% w/v solution), and aliquots (3×1 ml) were added by pipette to a small Petri dish. After 7 days of cultivation at 37 °C in a humidified atmosphere containing $CO_2(g)$ (5% v/v), aqueous citric acid (five drops of a 5% w/v solution) was added, and the dishes were incubated at 4 °C to be stiffed. The colonies (>40 cells), large clusters (20–40 cells), and small clusters (<20 cells) in the dish were then quantitated.

2.10. Assay of immunogenic activity

The immunogenicity of ribonucleases in mice was assessed with a non-competitive ELISA test (Tahara et al., 1996). The wells of a microtiter plate were coated with ONC, BS-RNase, or RNase A (25 μg), and then washed. Antisera from mice

Table 1
Aspermatogenic activity of ribonucleases injected into left testis of mice[a]

Ribonuclease	No. of mice	Index mass of testes ($\pm$S.E.M.)		Width of spermatogenic layers of testes (μm$\pm$S.E.M.)		Diameter of seminiferous tubules of testes (μm$\pm$S.E.M.)		Degree of aspermatogenesis
		Injected testes	Non-injected testes	Injected testes	Non-injected testes	Injected testes	Non-injected testes	
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 ± 6	156 ± 6	0
BS-RNase	6	19 ± 8[b]	32 ± 10	36 ± 3[c]	62 ± 8	139 ± 13[c]	165 ± 24	2–3
ONC	5	9 ± 3[c]	41 ± 6	total destruction	42 ± 11[c]	total destruction	163 ± 24	4

[a] Mice were examined 10 days after the injection of a ribonuclease (100 μg).

[b] $P < 0.05$.

[c] $P < 0.01$.

treated with a ribonuclease and control sera from mice injected with PBS were diluted serially in wells, and the plates were incubated at 37 °C for 2 h. The SwAMPX (Swine anti-mice IgG with peroxidase; USOL, Prague, Czech Republic) conjugate diluted 1:1000 was added, and the resulting solution was incubated for 2 h. The substrate solution was then added, and the enzymatic reaction was stopped after 20 min by the addition of aqueous H_2SO_4 (4 N). The optical density of the solutions was measured at 450 nm using a spectrophotometer (Titertek, Uniskan, Flow Laboratories, Irvine, UK). Serum test results were defined as positive when the optical density of the serum tested was found to be at least 3 S.E.M. (standard error of the mean) higher than that of mice injected with PBS.

3. Results

3.1. Aspermatogenic activity

Intratesticular injection of ONC (100 μg) into the left testis of mice resulted in the destruction of tubular structures and cessation of spermatogenesis (Table 1). BS-RNase evoked less strong degeneration, as testicular tubules were not destroyed. The same dose of RNase A did not exert any aspermatogenic activity. The destruction of testes was observed 20 days after the injection of ONC or BS-RNase into both testes (Table 2). In comparison to BS-RNase, mice injected with ONC suffered a greater decrease in testicular mass and degeneration of testicular tissue structure. The seminiferous tubules and spermatogenic cells were necrotized and invaded by polymorphonuclears,

monocytes, plasma cells, and lymphocytes. The number of fibroblasts and fibrocysts increased greatly (Table 2, Fig. 2). In most tubules, the spermatogenic cells were totally absent (Fig. 3). The spermatogonia vacuolysed and lost their mitotic activity. In most tubules, the spermatocysts degenerated fully. Sertoli cells were also destroyed. The ligament parts of testes spread rather than the tubules, but Leydig cells remained unchanged (Figs. 4 and 7). During these first 20 days after injection, the mass of the epididymides and seminal vesicles did not decrease substantially. In mice injected with ONC, the heads of epididymides were without sperm (Fig. 5), whereas in mice injected with BS-RNase, the number of sperm merely decreased (Table 2).

The histological situation was nearly identical 40 and 60 days as 20 days after ONC injection. The testicular tissue was destroyed fully in most tubules, which lacked any original testicular tissue cells, with the exception of Leydig cells, which were constantly present in the ligament parts of tissue. In some tubules, however, some spermatogenic cells were present. The index mass of epididymides decreased significantly (Table 2) but without any distinct change in the epithelial part. Although the mass of the seminal vesicles appeared to decrease, this decrease was not significant, and the thickness of epithelial layers was normal (Table 2, Fig. 6). In contrast, the testes of mice injected with BS-RNase (100 μg) were degenerated 20 days after the injections, but later the width of spermatogenic layers and diameter of seminiferous tubules returned to normal (Table 2). Eighty and 100 days after the injection of ONC, the spermatogenic layers disappeared in testicular

Table 2
Aspermatogenic activity of ribonucleases injected into both testes of mice[a]

Ribonuclease injected	No. of mice	No. of days after injection	Index mass of testes ($\pm$S.E.M.)	Width of spermatogenic layers of testes (μm$\pm$S.E.M.)	Diameter of seminiferous tubules of testes (μm$\pm$S.E.M.)	Index mass ($\pm$S.E.M.)		No. of sperms per mm^2 of epididymides
						Epididymides	Seminal vesicles	
PBS (control)	5	20	90$\pm$8	63$\pm$9	163$\pm$11	12$\pm$2	76$\pm$12	23$\pm$4
RNase A	5	20	90$\pm$12	62$\pm$5	167$\pm$13	13$\pm$3	60$\pm$16	not determined
RNase A	5	60	86$\pm$7	65$\pm$5	163$\pm$10	13$\pm$4	59$\pm$8[b]	21$\pm$9
BS-RNase	5	20	41$\pm$9[c]	33$\pm$17	140$\pm$6[c]	10$\pm$3	90$\pm$6[b]	4$\pm$9[c]
BS-RNase	5	40	62$\pm$15[c]	55$\pm$16	155$\pm$12	12$\pm$2	80$\pm$11	<1[c]
BS-RNase	5	60	84$\pm$11[c]	63$\pm$9	164$\pm$7	11$\pm$2	82$\pm$10	9$\pm$3[c]
BS-RNase (100+250 μg)	5	60	38$\pm$10[c]	34$\pm$7	120$\pm$9[c]	9$\pm$6[b]	80$\pm$9	0[c]
ONC	7	20	24$\pm$15[c]	14 testicles: 0	2 testicles: 118$\pm$18 12 testicles: 0	11$\pm$5	61$\pm$13	0[c]
ONC	9	40	25$\pm$9[c]	6 testicles: 36$\pm$14 12 testicles: 0	5 testicles: 127$\pm$51 13 testicles: 0	7$\pm$3[c]	62$\pm$19	<1[c]
ONC	5	60	31$\pm$23[b]	4 testicles: 34$\pm$11 6 testicles: 0	5 testicles: 121$\pm$48 5 testicles: 0	8$\pm$2[c]	64$\pm$18	0[c]
ONC	4	80	28$\pm$29[c]	1 testicle: 42 7 testicles: 0	4 testicles: 108$\pm$32 4 testicles: 0	6$\pm$2[c]	74$\pm$19	0[c]
ONC	5	100	16$\pm$12[c]	10 testicles: 0	6 testicles: 111$\pm$30 4 testicles: 0	6$\pm$1[c]	78$\pm$11	0[c]
ONC (100+25 μg)	6	100	8$\pm$3[c]	10 testicles: 0	12 testicles: 0	5$\pm$1[c]	78$\pm$6	0[c]

[a] Mice were examined after the intratesticular injection of a ribonuclease (100 μg).
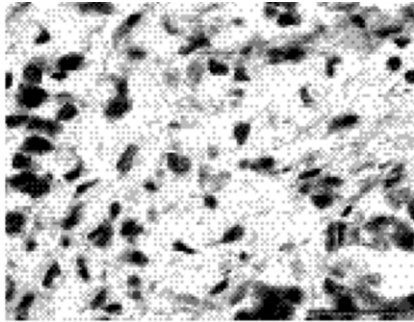[b] $P < 0.05$.
[c] $P < 0.01$.

Fig. 2. Effect of ONC on mouse seminiferous tubules and spermatogenic cells. Image was obtained 10 days after intratesticular injection of ONC (100 μg). Bar, 10 μm.

Fig. 4. Effect of ONC on the spermatogenic cells within mouse seminiferous tubules. Image was obtained 10 days after intratesticular injection of ONC (100 μg). Bar, 10 μm.

tubules. In only one testicle from four mice did the spermatogenic cell reach a width of 42 μm, whereas the other seven testicles of these four animals had tubules without cells. The seminiferous tubules of four testes of measurable diameter had an average diameter of 108 μm, but the spermatogenic cells were largely degenerated, even if some spermatogonia were mitotically active. A similar picture was apparent in the tubules of mice 100 days after ONC injection. When six mice were

reinjected with 25 μg of ONC 80 days after an initial injection of 100 μg, the testes were degenerated fully after 20 days (Table 2). When five other mice were reinjected with 100 μg of ONC, four died within 1 h.

Leydig cells were present in the testicular tissue of all animals studied by histology from 20 to 100 days after the injection of ONC or BS-RNase. They were also in fully ligamented tissues of mice injected twice by ONC or BS-RNase, even if no
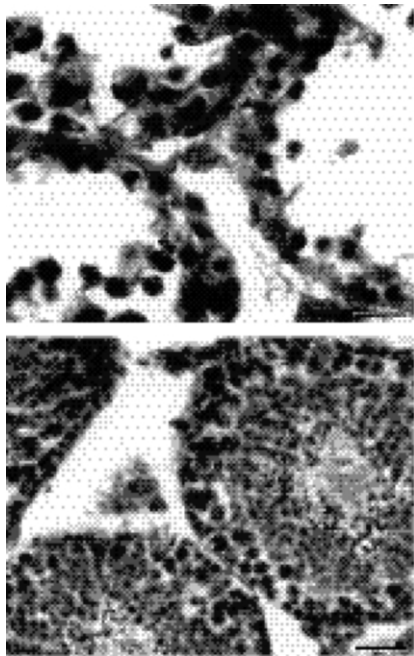

Fig. 3. Effect of ONC on mouse seminerous tubules. Images were obtained 10 days after intratesticular injection of (A) ONC (100 μg) or (B) PBS. Bar, 10 μm.
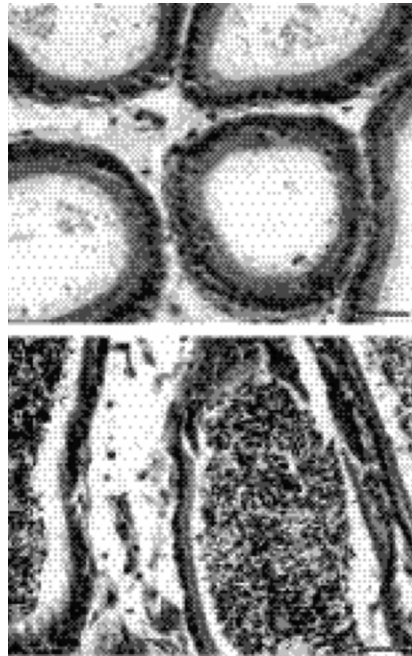

Fig. 5. Effect of ONC on the heads of mouse epididymides. Images were obtained 40 days after the intratesticular injection of (A) ONC (100 μg) or (B) PBS. Bar, 10 μm.

Fig. 6. Effect of ONC on the epithelial layers of mouse seminal vesicles. Image was obtained 60 days after the intratesticular injection of ONC. Bar, 10 μm.
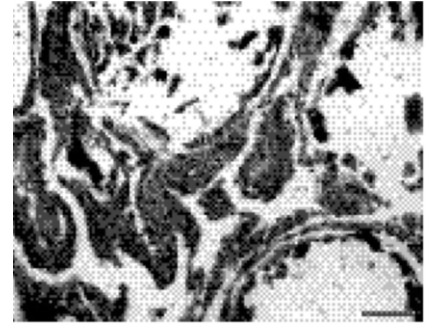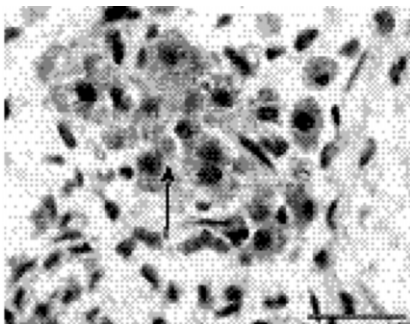

Fig. 7. Effect of ONC on mouse Leydig cells in the small islet (arrow). Image was obtained on Day 100 after intratesticular injections of ONC on Day 0 (100 μg). Bar, 10 μm.

ligamentation was observed in animals injected with BS-RNase. Leydig cells were situated mainly in the small islets, in which 5–21 cells were observable (Fig. 7).

Similar histological changes were observed in two rats 100 days after the injection of ONC (1000 μg) in both testes (Fig. 8). No spermatogenic

Fig. 8. Effect of ONC on the spermatogenic cells within rat seminiferous tubules. Image was obtained 100 days after intra-testicular injection of ONC (1000 μg). Bar, 10 μm.

cells were present in seminiferous tubules. The mass of these testicles was 0.90 ± 0.13 g (two rats). In contrast, the mass of rat testicles injected with a saline solution was 4.9 ± 2.1 g (two rats). Testosterone production by Leydig cells was assayed by quantitating this hormone in the serum of injected rats. The testosterone level of ONC-injected rats was 1.93 ± 0.25 pg/ml, and that of saline-injected rats was 2.10 ± 0.57 pg/ml. These values were not distinguishable.

3.2. Embryotoxic activity

A strong embryotoxicity of ONC in vitro was apparent from the data listed in Table 3. RNase A was not toxic at a concentration of 100 μg/ml. The same concentration of BS-RNase was only slightly embryotoxic, whereas ONC was completely toxic to embryos at 10 and 100 μg/ml.

The embryotoxicity of mice pregnant for 1–5 days was similar to the in vitro embryotoxicity data, as listed in Table 4. RNase A did not evoke embryotoxicity. Intraperitoneal injection of BS-

Table 3
Embryotoxic activity of ribonucleases in vitro[a]

Ribonuclease	No. of mouse embryos	No. of embryos at cell stage			
		Blastocysts	Expanded blastocysts	Total blastocysts	Blastocysts (%)
PBS (control)	25	10	8	18	72
RNase A (100 μg/ml)	9	1	5	6	67
BS-RNase (100 μg/ml)	10	1	0	1	10
ONC (1 μg/ml)	21	7	8	15	74
ONC (10 μg/ml)	27	0	0	0	0
ONC (100 μg/ml)	32	0	0	0	0

[a] Mouse embryos were incubated with a ribonuclease for 72–96 h at 37.5 °C.

Table 4
Embryotoxic activity of ribonucleases in mice[a]

Ribonuclease	No. of mice	No. of days from mating to first	Ribonuclease injected (μg)		No. of pregnant females		No. of living embryos (±S.E.M.)	
			At one injection	Total	Total	%	Per pregnant female	Per injected female
PBS (control)	10	1–5	0	0	8	80	9±2	7±4
PBS (control)	10	6–10	0	0	9	90	8±2	8±3
BS-RNase	14	1–5	1000	3000	2	14	8±3	1±3[b]
BS-RNase	15	6–10	1000	3000	3	20	7±4	2±1[b]
ONC	15	1–5	20	20	6	40	10±6	4±4[b]
ONC	7	1–5	200	200	0	0	0	0[b]
ONC	5	6–10	200	200	1	20	9±5	2±4[b]

[a] Embryos were examined 4 days after injection.
[b] $P < 0.01$.

RNase (3×1000 μg) was significantly embryotoxic. Injection of ONC (1×200 μg) was fully embryotoxic in females pregnant 1–5 days, and partly embryotoxic in females pregnant 6–10 days. Injection of ONC (1×20 μg) was also partly embryotoxic in females pregnant 1–5 days (Table 4).

3.3. Antitumoral activity

ONC had a more pronounced effect on the proliferation of human ML-2 cells than did BS-RNase (Fig. 9). The difference was most evident
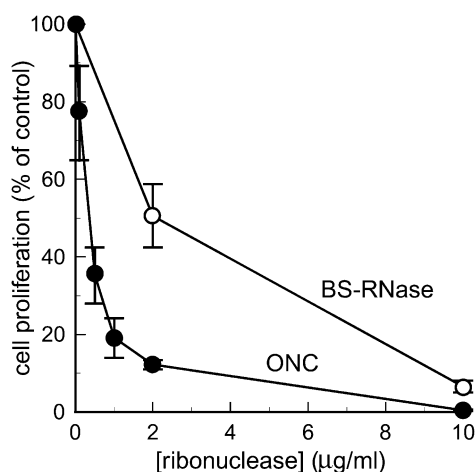
in the presence of low concentrations of ribonuclease. The concentration producing 50% inhibition of cell growth (IC_{50}) for ONC and BS-RNase was 0.4 μg/ml and 2 μg/ml, respectively.

3.4. Immunosuppressive activity

ONC was more inhibitory than BS-RNase to the proliferation of lymphocytes in a mixed-lymphocyte culture (Fig. 10). An ONC concentration of 10 μg/ml completely inhibited lymphocyte proliferation, whereas a five-fold higher concentra-

Fig. 9. Effect of ONC and BS-RNase on the proliferation of human ML-2 cells. Cell proliferation was determined by incorporation of [6-³H]thymidine into cellular DNA after a 44-h incubation with a ribonuclease. Each value is the mean (±S.E.) of the three cultures containing a ribonuclease compared with that of untreated control cells.
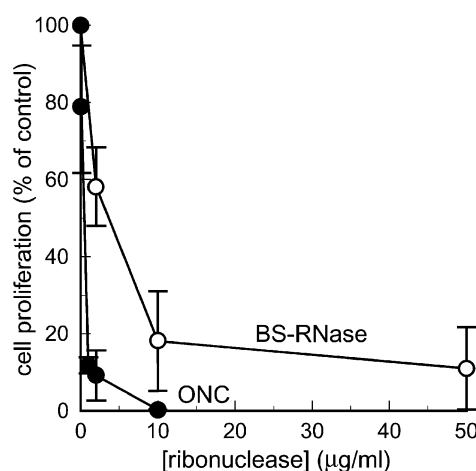

Fig. 10. Effect of ONC and BS-RNase on the proliferation of human lymphocytes in a mixed lymphocyte culture. Cell proliferation was determined by incorporation of [6-³H]thymidine into cellular DNA after a 6-day incubation with a ribonuclease. Each value is the mean (±S.E.) of the three cultures containing a ribonuclease compared with that of untreated control cells.
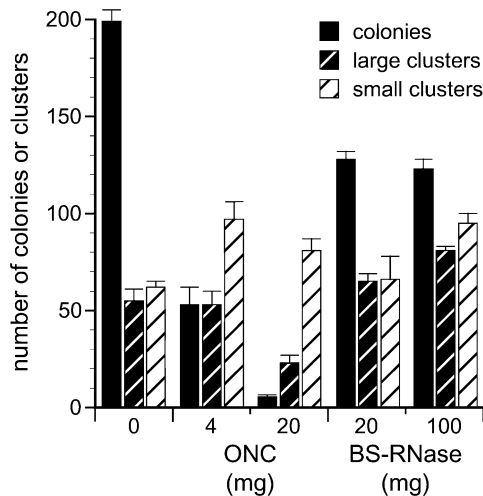
Fig. 11. Effect of ONC and BS-RNase on the CFU-GM of human bone marrow cells. Colonies (>40 cells), large clusters (20–40 cells), and small clusters (<20 cells) were quantitated after a 7-day incubation with a ribonuclease.

tion of BS-RNase produced $<90\%$ inhibition of lymphocyte proliferation.

3.5. Colony-forming activity with bone marrow cells

ONC but not BS-RNase greatly diminished the colony-forming activity of human bone marrow cells. In previous work (Soucek et al., 1986; Cinatl et al., 1999), BS-RNase at 50 μg/ml was shown to have no harmful effect on bone marrow cells gained from normal donors. Here, ONC and BS-RNase were assayed similarly at concentrations demonstrated to be highly effective at inhibiting tumor cell proliferation (i.e. 4 and 20 μg/ml of ONC; 20 and 100 μg/ml of BS-RNase). As shown in Fig. 11, the number of colonies exposed to BS-

RNase was only slightly lower than that in control cultures. In contrast, ONC at 4 μg/ml caused ca. 75% inhibition of colony-forming activity, and ONC at 20 μg/ml caused nearly complete inhibition.

3.6. Immunogenic activity

As indicated by antibody production in mice, the immunogenicity of ONC is less than that of BS-RNase, but greater than that of RNase A (Table 5). The injection of a second, small quantity ONC into the testes of mice did, however, increase the production of antibodies nearly to the level engendered by BS-RNase.

4. Discussion

Spermatogenesis can be especially vulnerable to the cytotoxicity of ribonucleases. The intratesticular injection of BS-RNase into mice, rats, and rabbits, as well as its subcutaneous, intraperitoneal, or intrascrotum injection, evokes aspermatogenesis that can revert to normal (Dostál and Matousek, 1973; Matousek and Grozdanovic, 1973; Matousek et al., 1978; Matousek, 1994). RNA levels in testes decrease but return to normal 50 days after the subcutaneous injection of BS-RNase, whereas no decrease in RNA levels in liver and kidney tissues can be detected after injection (Matousek et al., 1973). After the administration of BS-RNase to rats, no Leydig cells are damaged and testosterone levels remain constant (Hlinák et al., 1981).

In marked contrast, the degeneration of spermatogenic cells in mice is dramatic and irreversible after the intratesticular injection of ONC (Figs. 2, 3 and 5, Tables 1 and 2). Nonetheless, Leydig cells are not destroyed, as indicated by the production of testosterone in the blood serum of rats

Table 5
Titer of anti-ribonuclease antibodies in mice

Ribonuclease injected (μg)	No. of mice	Titer of antibodies with ribonuclease antigen		
		RNase A	BS-RNase	ONC
PBS (control)	5 male	0	0	0
RNase A (100)	5 male	10–80	0–10	0–10
BS-RNase (100)	11 male	320–640	1600–3200	160–1600
BS-RNase (100+250)	5 male	320–1280	6400–12 800	160–640
ONC (20)	15 female	0–20	0–20	0–40
ONC (100)	35 male	40–60	40–320	80–320
ONC (100+25)	6 male	160–640	1600–3200	1600–6400
ONC (200)	12 female	40–320	40–160	80–3200

injected by ONC. Nor is there a change in the epithelial layers of seminal vesicles in mice (Fig. 6). The intratesticular injection of ONC into testes is accompanied by the invasion of polymorphonuclear cells, monocytes, and lymphocytes (Fig. 2). This drastic degeneration is similar to the complete ischemia of testes in mice, guinea pig, and rabbit (Matousek, 1983). These effects are not apparent after the injection of BS-RNase.

The destruction of spermatogenic cells in the testes of experimental animals by ONC portends the non-surgical cessation of sperm production with retention of sexual activity. The administration of ONC during cancer chemotherapy is unlikely to evoke such a strong antitesticular effect. Nevertheless, it would be desirable to test the spermatogenic effect of ONC injected in animals subcutaneously or intraperitoneally. Repeated injections of ONC could be accompanied by the toxicity manifested in our experiments, as a second intratesticular injection of ONC (100 μg) evoked the death of four of five mice (data not shown). Similar toxic effects (e.g. ataxia, muscular rigidity, and tremor leading to death) have been reported by others (Newton et al., 1994).

The embryotoxic activity of ONC is also much greater than that of BS-RNase, both in vitro (Table 3) and in vivo (Table 4). The embryotoxic activity of BS-RNase was first noted 30 years ago (Matousek, 1973b). At that time, the known link between embryonic particles and tumor development (Hirsfeld and Halber, 1932) inspired the characterization of the antitumoral activity of BS-RNase (Matousek, 1973b; Stanek and Matousek, 1976; Cinatl et al., 1977). The correlation of embryotoxic and antitumoral activity was confirmed with recombinant BS-RNase (Kim et al., 1995b,c,a), which was also detrimental to oocyte maturation (Slavík et al., 2000).

Interestingly, the embryotoxic activity of a ribonuclease and its antitumoral activity need not be linked. Trimers and tetramers of RNase A display antitumoral activity but have little embryotoxicity (Matousek et al., 2003). Likewise, RNase A conjugated to poly [*N*-(2-hydroxypropyl) methacrylamide] and polyethylene glycol have antitumoral activity with little or no embryotoxic activity (Ulbrich et al., 2000; Matousek et al., 2002; Soucek et al., 2002).

The antitumoral activity of ONC in vitro is greater than that of BS-RNase (Fig. 9). Likewise, ONC has a more deleterious effect on the prolif-

eration of human lymphocytes stimulated by a mixed lymphocyte culture (Fig. 10). This effect could lessen the innate immune response in vivo, and thus could be disadvantageous for the use of ONC as a chemotherapeutic. Another disadvantage of ONC in comparison with BS-RNase is its toxicity for human bone marrow cells. In previous work, BS-RNase was shown to be non-toxic for human bone marrow cells (Soucek et al., 1986) and to produce only a small reduction in colony-forming activity in pigs (Matousek et al., 2001). Here, the toxicity of BS-RNase for normal human bone marrow cells was again negligible, whereas the cytotoxicity of much lower doses of ONC was severe (Fig. 11).

The immunogenicity of BS-RNase is greater than that of ONC (Table 5). The greater immunogenicity of BS-RNase could arise from its larger size as well as its two-fold higher monomer valency (Fig. 1) (Tam, 1988). The higher production of antibodies against BS-RNase is known to decrease its aspermatogenic activity in mice, but only slightly (Matousek, 1994). Repeated injections of ONC did increase its immunogenicity. In contrast to BS-RNase and ONC, RNase A is virtually without immunogenic activity.

Biochemical basis for the relative cytotoxicity of ribonucleases. In general, the cytotoxicity of pancreatic-type ribonucleases decreases in the order: ONC > BS-RNase ≫ RNase A. What is the biochemical basis for this order? Of these three ribonucleases, RNase A alone is bound tightly by the ribonuclease inhibitor protein (RI) that is endogenous to the cytosol of mammalian cells (Hofsteenge, 1997; Shapiro, 2001). The RI-RNase A complex has an equilibrium dissociation constant of $K_d = 10^{-14}$ M (Lee et al., 1989; Vicentini et al., 1990), making the RI-RNase A interaction one of the strongest known non-covalent interactions in biology. Variants of RNase A or its human homolog that can evade RI without losing ribonucleolytic activity or conformational stability are cytotoxic (Leland et al., 1998; Bretscher et al., 2000; Leland et al., 2001; Haigis et al., 2002; Dickson et al., 2003), indicating that the ability to evade RI is an essential attribute of a cytotoxic ribonuclease.

In marked contrast to RNase A, neither ONC nor BS-RNase has a demonstrated affinity for RI (Murthy and Sirdeshmukh, 1992; Wu et al., 1993; Haigis et al., 2003). Accordingly, both wild-type ribonucleases can manifest fully their ribonucleo-

lytic activity in the cytosol of a mammalian cell. Interestingly, mammalian ribonucleases (such as BS-RNase and RNase A) have 10^3-fold more ribonucleolytic activity than do amphibian ribonucleases (such as ONC) (Lee and Raines, 2003). Why then is ONC more cytotoxic than BS-RNase? We note two differences that could be responsible for the greater cytotoxicity of ONC.

First, BS-RNase is much larger than ONC (Fig. 1). In addition to being a homodimer, each monomeric subunit of BS-RNase (124 residues) is larger than ONC (104 residues). Gaining access to cellular RNA requires an extracellular protein to cross a lipid bilayer. The 2.4-fold greater size of BS-RNase could hinder its ability to cross a lipid bilayer and thereby diminish its cytotoxic activity. This proposal seems to be at odds, however, with the notable antitumoral activity of long polymers that are decorated with RNase A (Ulbrich et al., 2000; Matousek et al., 2002; Soucek et al., 2002).

Second, BS-RNase monomers bind tightly to RI (Murthy and Sirdeshmukh, 1992) and are not cytotoxic (D'Alessio et al., 1997). Although the subunits of dimeric BS-RNase are linked by two disulfide bonds, these bonds are vulnerable to reduction in a reducing environment such as the cytosol of a mammalian cell (Hwang et al., 1992). The reduction of the intersubunit disulfide bonds of BS-RNase would create monomers that could suffer inhibition by RI. Indeed, covalent modification enables BS-RNase monomers to evade RI and become cytotoxic, at least to spermatogenic layers (Matousek et al., 1997). This proposal appears to be undermined, however, by our previous report that a dimeric BS-RNase variant in which the monomers are linked by a non-reducible cross-linker has cytotoxic activity that is comparable, but not greater, than that of BS-RNase (Kim et al., 1995b).

5. Conclusion

We have shown that both ONC and BS-RNase demonstrate cytotoxicity in a broad variety of in vitro and in vivo assays. In general, however, we find ONC to be the more potent cytotoxin. ONC has advanced to Phase III in human clinical trials for the treatment of malignant mesothelioma. The success of ONC in the clinic and the data reported herein establish ONC as the most cytotoxic homolog of RNase A.

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