Effect of wheat leaf ribonuclease on tumor cells and tissues

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The antiproliferative and antitumor effect of wheat leaf ribonuclease was tested *in vitro* on the human ML-2 cell line and *in vivo* on athymic nude mice bearing human melanoma tumors. The antiproliferative activity of this plant ribonuclease was negligible in comparison with bovine seminal ribonuclease. In the experiments *in vivo*, a significant decrease of the tumor size, however, was observed in the mice treated with wheat leaf ribonuclease (27 kDa) compared with the control RNase A and polyethylene glycol. In nude mice injected intratumoraly with wheat leaf ribonuclease, the tumor size decreased from 100% in the control mice to 39% in treated mice. In the mice treated with polyethylene glycol-conjugated wheat leaf ribonuclease, the tumor reduction was observed from 100 to 28%, whereas in counterparts treated with polyethylene glycol-conjugated bovine seminal ribonuclease the tumor inhibition was reduced from 100 to 33%. Certain aspermatogenic and embryotoxic activity of wheat leaf ribonuclease and bovine seminal ribonuclease also appeared, but was lower in comparison with the effect of onconase. Mutual immunological cross-reactivity between wheat leaf ribonuclease antigens on one side and animal RNases (bovine seminal ribonuclease, RNase A, human HP-RNase and onconase) on the other side proved a certain structural similarity between animal and plant ribonucleases. Immunogenicity of wheat leaf ribonuclease was weaker in comparison with bovine seminal ribonuclease (titer of antibodies 160–320 against 1280–2560 in bovine seminal ribonuclease). Interestingly, immunosuppressive effect of wheat leaf ribonuclease tested on mixed lymphocyte culture-stimulated human lymphocytes reached the same level as that of bovine seminal RNase. The antibodies against wheat leaf ribonuclease produced in the injected mice did not inactivate the biological effect of this plant RNase *in vivo*. This is probably the first paper in which plant ribonuclease was used as antiproliferative and antitumor drug against animal and human normal and tumor cells and tissues in comparison with animal ribonucleases. *Anti-Cancer Drugs* 17:815–823 © 2006 Lippincott Williams & Wilkins.

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Introduction

Animal RNases with some biological, mainly antiproliferative, effects [1–3] have their counterparts in plants. Plant ribonucleases designated as S-RNases are involved in activities such as the processing and turnover of cellular RNA. Some of them are important components of self-incompatibility functions in plants, whereas others are not associated with their incompatibility [4–6]. These RNases are involved in physiological and developmental signals, such as nutrient deficiency, attack of pathogens or heat shock, as the means of reducing RNA turnover until recovery. Senescence and wounding of plant tissues are known to induce rapid inclusion of RNase activities into these processes such as defence of plants against RNA viruses and programmed cell death [4,7]. It is known from the literature that only isolated thermolabile heterodimeric ribonucleases have been studied on antifungal and antiproliferative activities. Three research groups isolated these types of RNases from different sources, i.e. roots of Sanchi ginseng (*Panax notoginseng*), immunotoxin of Moschating from mature seeds of pumkin (*Cucurbita moschata*), as well as RNase from mature seeds of oriental arbovitae (*Biota orientalis*) [8–10]. On the basis of this limited information, we decided to isolate RNases from leaves of higher plants, and to study their biologic and antitumor effects on animals.

Mainly, the plant RNases of S-like type might be compared with animal RNases for studies of their biological effects on animal cells and tissues. They have molecular masses between 21 and 29 kDa, and are considered to be secretory proteins. Three RNase activities were characterized in wheat leaves [11], while two of them were found to be of a neutral type. One of

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them was approximately 27 kDa in size and was inhibited by salt or $MgCl_2$, whereas the other one pertained to a group of RNases where the activity is stimulated by salt or $MgCl_2$ [11]. This ribonuclease is not studied in this paper, even its activity due to the stimulation by salt concentration could have more antiproliferative activity in comparison with the 27-kDa RNase enzyme. Both these ribonucleases have a similar molecular mass to animal bovine seminal ribonuclease (BS-RNase) with its antiproliferative, antitumor and other biological activities [1–3]. The most soluble RNase activity in plant cells is situated mainly in vacuoles and endoplasmic reticulum, the other outside cells [4–6]. Apart from these two neutral wheat leaf RNases, DNase also was defined in wheat leaves [11,12]. The antitumor effect of pine pollen nuclease on nude mice was already presented (Matousek *et al.*, 7th International Meeting on Ribonucleases, 16–20 June, 2005, Stará Lesná, Slovak Republic, Abstracts, p. 81).

Material and methods

Materials

Bovine pancreatic ribonuclease (RNase A) was purchased from MP Biochemicals, Irvine, California, USA. BS-RNase was prepared from bull seminal plasma using the method published previously [13,14]. Onconase (ONC) and human pancreatic RNase (HP-RNase) were produced in *Escherichia coli* by recombinant DNA technology [15]. Both of them were a kind gift from Professor Ronald T. Raines and Dr Eugene Lee (University of Wisconsin, Madison, USA). Activated derivatives of polyethylene glycol (PEG; MW 5129) were purchased by Sherwater Polymers Hundville, Alabama, USA. Plastic microtiter plates (Gamma, Ceske Budejovice, Czech Republic) were used for determination of antibodies. The SwAMPx (swine anti-mouse IgG with peroxidase) produced by Sevapharma (Prague, Czech Republic) was used for antibody titration measured with a Titertek Uniskan (Flow Laboratories, Irvine, UK).

Isolation and purification of wheat leaf neutral RNase

Wheat leaves taken about 30 days after anthesis and kept under frozen conditions were ground in liquid nitrogen supplemented with 10% of polyvinylpyrolidon. For every 50 g of ground tissue, 200 ml of buffer A (10 mmol/l Tris, pH 7.5, 5 mmol/l ethylene diaminetetraacetic acid and 1 mmol/l α-toluensulfonylfluoride-phenylmethylsulfonyl fluoride) was added and homogenized by an IRA-Werke S25N-25F dispersing tool. Following centrifugation at 6500 *g* for 30 min at 4°C, filtration through eight layers of cheesecloth was done and protein fractions were precipitated from crude extract using ammonium sulfate. The 80% ammonium sulfate precipitate containing neutral RNase activities was dialysed in buffer A. After centrifugation, the sample was loaded on a DEAE-Sephacell column previously equilibrated with buffer A [12]. Fraction 27 kDa containing neutral RNase activity was determined using RNA [12] and its purity was controlled by electrophoresis [11,16] see Fig. 1. The eventual effect of glycosidases in biological studies was performed by testing β-D-glucoronidase, α-D-glucosidase, β-D-glucosidase, α-D-manosidase, β-D-galactosidase and α-D-galactosidase [17].

Wheat leaf ribonuclease intermolecular conjugation to polyethylene glycol

As a desired point of enzyme attachment, lysine and high-reactive ester (*N*-hydroxyl succinidyl derivative) of PEG were used because the linkage between amino groups of enzyme and PEG provides (under mild reaction condition) a stable conjugate relatively resistant to hydrolytic cleavage [18]. PEG was coupled under nitrogen atmosphere in 0.1 mol/l sodium phosphate buffer pH 7.4 at 4°C for 35 min. After this reaction, the nonreacted substances were removed by ultrafiltration through an Amicon PM-50 membrane and purified by size-exclusion chromatography on Sephadyl S-300 in the buffer mentioned above. Eluted peaks were UV-photometrically evaluated and their protein contents were determined [19].

Antiproliferative activity tested *in vitro* on a cell culture

The cell line ML-2 (derived from human myeloid leukemia) was used for testing RNase antiproliferative activity as described previously [20–22]. The total of 2×10^5 cells in 0.2 ml RPMI 1640 medium supplemented with fetal calf serum (10% v/v) was established in a microtiter plate (Nunc, Gamma, Ceske Budejovice Czech Republic, FB type) and cultivated in a humidified atmosphere containing 5% of CO_2 for 48 h. Simultaneously, the known concentration of RNase was added to each triplicate culture. Four hours before the termination of cultivation, the samples were pulsed with 24 kBq of [6-^{3}H]thymidine (specific activity 980 GBq/mmol). Then cells were collected with a Scatron cell harvester and radioactivity was measured in a β-counter (Beckman). The mean values of the triplicates containing a particular ribonuclease were compared with those of the untreated control cells and expressed as percentage of inhibition.

Immunosuppressive activity tested *in vitro* on human lymphocytes

Immunosuppressive activity of wheat leaf ribonuclease (WLN-RNase), RNase A and BS-RNase on human lymphocytes stimulated in mixed lymphocyte culture was assessed as described previously [21]. Lymphocytes from two unrelated donors were mixed and cultivated in a humidified atmosphere containing 5% of CO_2 at 37.0°C in RPMI 1640 medium supplemented with mixed human AB serum (10% v/v). The known concentration of studied ribonucleases was added at the beginning of the experiment and the cell mixture was incubated for 6 days. The pulsation with [6-^{3}H]thymidine and calculation of the inhibitory effect were carried out as described above in the previous section.

Antitumor activity tested on nude mice

Antitumor activity of free WLN-RNase, RNase A, BS-RNase and WLN-RNase conjugated to PEG polymer was tested on athymic female nude mice CD-1 weighing 18–20 g. The mice were housed under aseptic conditions in cages with bedding (SAWI Research) sterilized by irradiation and fed with sterile diet as referred previously [22]. Human melanoma was obtained from surgical specimens and pieces $3 \times 3\,mm^2$ were transplanted subcutaneously on the right flank of a mouse. Treatment of the mice started when the area of the transplanted tumor reached $5 \times 5\,mm^2$. RNase preparations were injected three times a week usually for 3 weeks. The daily dose of conjugated preparations was calculated as micrograms of the free RNase per each 20 g of body weight. The tumor volume was determined twice a week using a slide caliper (measuring length × width × depth) [23].

Spermatogenic toxicity in mice

Aspermatogenic effect of the isolated WLN-RNase, RNase A and BS-RNase was determined as described previously [14]. Adult male ICR mice were injected with 100 μg of WLN-RNase, RNase A and BS-RNase dissolved in phosphate-buffered saline (PBS). The mice were also injected with 100 μg WLN-RNase dissolved in immune mice anti-WLN-RNase serum or with normal nonimmune mice serum into the left testes and killed after 10 days to determine the effect of immune antibodies on the biologic activity of injected WLN-RNase. The injected testes without epididymes were excised and histologically studied. Destructive effects on the testes were detected by the decrease in the width of spermatogenic layers and the diameter of seminiferous tubules. Between 50 and 60 tubules from the central part of the testes were measured under a microscope equipped with micrometer scale. Additionally, in order to investigate the potential of the WLN-RNase to be distributed within the body and to reach its site of action, this enzyme in comparison with RNase A and BS-RNase was administered intraperitoneally at the dose of 100 μg once a week during 5 weeks, and compared with the effect of RNase A and BS-RNase. Degenerative effects of the testes were detected by the measurement of testes as described above.

Embryotoxic effect

The effect of WLN-RNase, RNase A, BS-RNase and ONC on the development of mouse embryos *in vitro* was assessed. Two-cell embryos of superovulated mice were flushed out of oviducts 36 h after mating. Embryos were cultured in CZB medium supplemented with bovine serum albumin (3 mg/ml) and with added ribonuclease at the dose of 10 or 100 μg/ml for 72–96 h at 37.5°C in humidified atmosphere containing 5% of CO_2. The development stage of embryos was evaluated by microscopy [24].

Immunogenicity determination

Immunogenicity of WLN-RNase, RNase A, BS-RNase, human pancreatic RNase CHP-RNase and ONC was determined as described previously [14]. A noncompetitive enzyme-linked immunosorbent assay test [25] was performed. Microtiter plate wells were coated with 25 μg of all studied ribonucleases. After washing the plates, antibodies from mice treated with the above-mentioned ribonucleases and control sera from mice injected with PBS were serially diluted in the wells and incubated at 37°C for 2 h. The SwAMPx (swine anti-mice IgG with peroxidase) conjugate 1:1000 was added and after 20 min of incubation in the substrate solution the reaction was stopped with addition of 4 N H_2SO_4. The antibody reaction was measured using photometry at 450 nm. The tests were defined as positive when optical density of the tested serum was found to be at least three standard errors of the mean (SEM) higher than that of the control mice injected with PBS [26].

Complex of wheat leaf ribonuclease with antibodies and its in-vivo studies on mice testes

The sera from mice injected by WLN-RNase exhibiting the titer of antibodies from 160 to 320 were pooled and used for antigen–antibody tests. Fifty microliters of pooled serums were mixed with 50 μg of isolated plant ribonuclease and tested *in vivo* by injecting of 10 μl of this mixture into left testes of five mature mice. The mice were killed after 10 days, and the width of spermatogenic layers and diameter of seminiferous tubules were histologically tested. Administration of WLN-RNase and normal mice serum or free WLN-RNase, BS-RNase and RNase A were involved as control tests [14].

Histology

All the animals injected with RNases were subjected to excision of testes and tumor enucleation. The small pieces of these tissues were consequently fixed in Bouin solution for histology examination. The fixed samples of tissues were embedded in paraffin blocks. Tissues slides (5 μm) were cut and stained with hematoxylin–eosin.

Statistical analysis

The results are presented as mean ± SEM. The data were analyzed statistically using Fisher's *t*-test.

Ethics

All the mentioned experiments adhered to ethical standards and were approved by the institutional committee (approval no. 3/04); all researchers handling experimental animals possess certificates from the Central Committee for Animal Welfare.

Results

Antiproliferative effect of wheat leaf ribonuclease on ML-2 tumor cell line *in vitro*

An inhibitory effect of the plant nuclease (WLN-RNase) tested on proliferation of human tumor cells ML-2 was compared with the effect of two animal RNases (BS RNase and ONC). As demonstrated in Fig 2, that plant enzyme displayed significantly lower antiproliferative activity on ML-2 tumor cells in comparison with both the BS-RNase and ONC, and did not differ significantly from that of RNaseA.

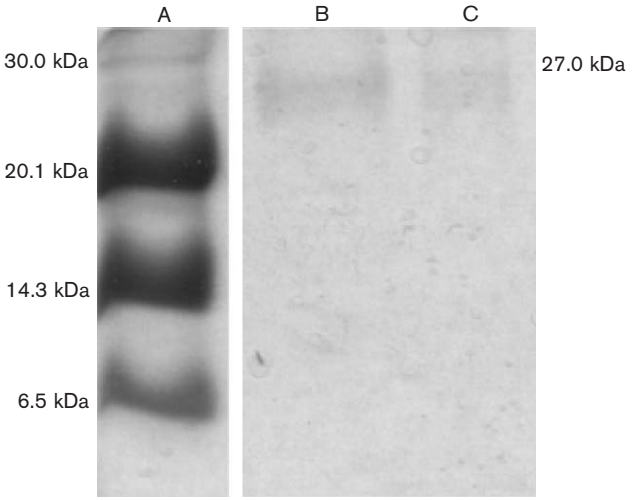
Immunosuppressive activity of wheat leaf ribonuclease *in vitro*

In contrast to the previous results mentioned above, WLN-RNase exerted a significant inhibitory effect on lymphocyte proliferation in the mixed lymphocyte culture, and was very close to that of BS-RNase and ONC, while the activity of RNase A was only very slightly pronounced (see Fig. 3). These experiments indicated functional similarity between WLN-RNase and BS-RNase that was also observed in the experiments with these two ribonucleases carried out on human melanoma *in vivo* or tested on testis degeneration or embryo cytotoxicity.

Antitumor activity of wheat leaf ribonuclease *in vivo* after the intratumoral application

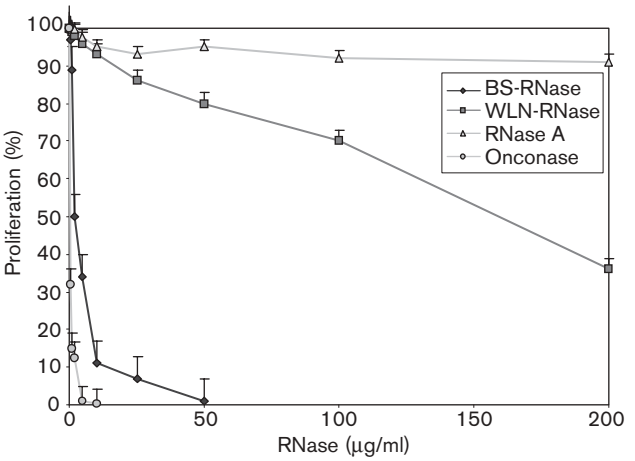
WLN-RNase, BS-RNase and RNase A (100 µg/mouse) were injected intratumorally in seven doses over a period of 20 days. The tumor volume was measured twice a week using a slide caliper. Antitumor activity of WLN-RNase in comparison with BS-RNase and RNase A is demonstrated in Fig. 4. Both the WLN-RNase and BS-RNase enzymes exerted a significant reduction of melanoma tumor growth in the nude mice after their intratumoral application, while injections of RNase A were ineffective. Nevertheless, the antitumor effect of BS-RNase was significantly more active than that of WLN-RNase. On the basis of our previous experience, RNase A was used in all experiments as the negative control. The application of PBS and PEG also showed a negative biological effect (Figs 4 and 5) when used.

Fig. 1



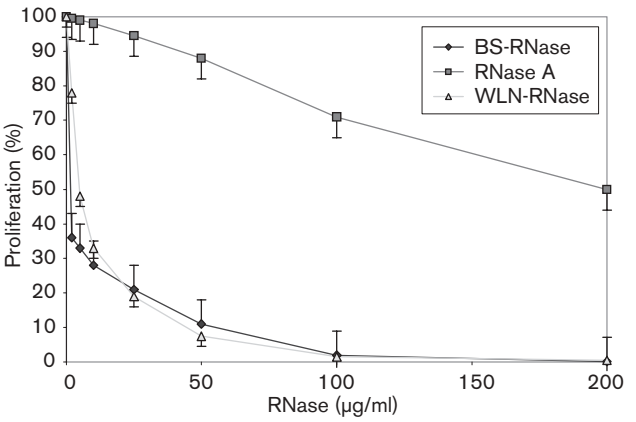
Purity of wheat leaf ribonuclease (WLN-RNase) was proved by discontinuous vertical sodium dodecyl sulfate electrophoresis on a 1-mm thick gel stained with Commassie Brillant Blue. From the left in column 1: four standards from the bottom: aprotinin 6.5 kDa, lysozyme 14.3 kDa, trypsin inhibitor 20.1 kDa, carbonic anhydrase 30.0 kDa; in column 2 WLN-RNase (sample A), in column 3 WLN-RNase (sample B).

Fig. 2



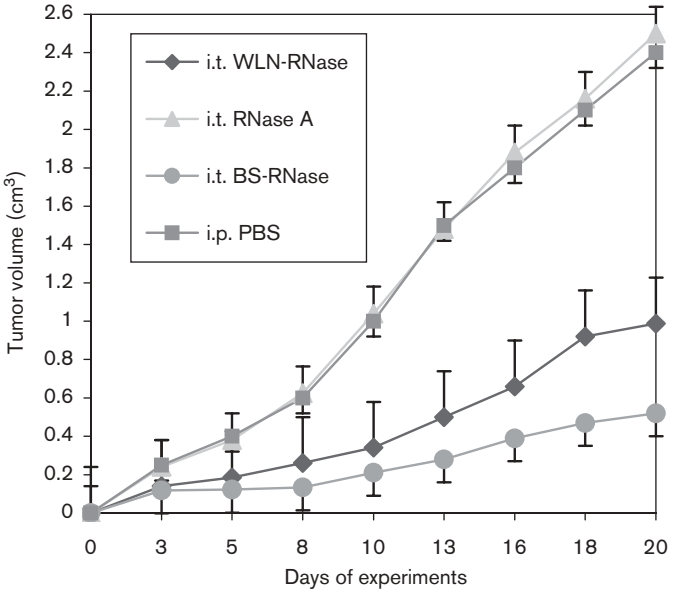
Effect of wheat leaf ribonuclease (WLN-RNase), bovine seminal ribonuclease (BS-RNase) and RNase A on the proliferation of human tumor cell line ML-2 in culture.

Fig. 3



Effect of wheat leaf ribonuclease (WLN-RNase) on the proliferation of human lymphocytes in a mixed lymphocyte culture in comparison with bovine seminal ribonuclease (BS-RNase) and RNase A.

Fig. 4

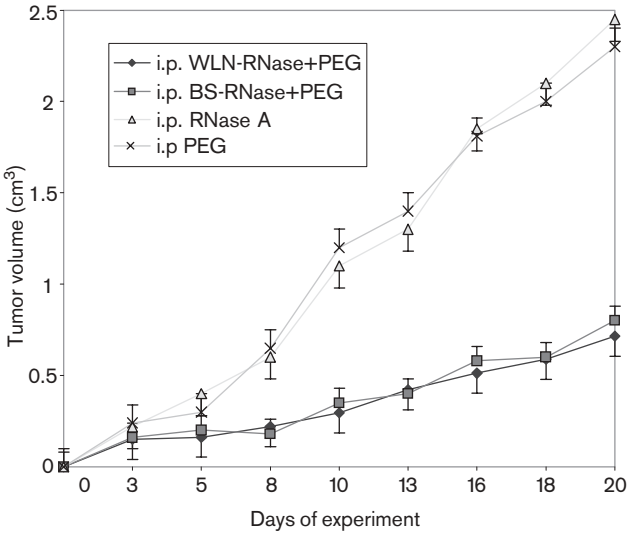


Human melanoma tumor growth in athymic *nu/nu* mice after wheat leaf ribonuclease (WLN-RNase) and bovine seminal ribonuclease (BS-RNase) injected intratumorally (i.t.) (PBS as a control). I.p., intraperitoneal.

Antitumor activity of polyethylene glycol-conjugated wheat leaf ribonuclease after intraperitoneal administration

In these experiments, WLN-RNase and BS-RNase conjugated to PEG were injected intraperitoneally in seven doses at 100 μg/mouse (amount of enzymes in the PEG complex has been about one-half) into the nude mice bearing a human melanoma tumor. As shown in Fig. 5, both enzymes exerted significant therapeutic efficiency. The tumor size in mice treated with PEG-conjugated WLN-RNase decreased from 100 (in the controlled group) to 28% and in the group of mice treated with PEG-conjugated BS-RNase from 100 to 33%. This effect of RNase conjugates was similar to that of free WLN-RNase and BS-RNase demonstrated above. RNase A demonstrated again as a negative control. The antitumor activity of WLN-RNase conjugated with PEG was also proved by histological examination (Fig. 6a). Tumor cells were degenerated in the whole tissue, the wall of a vein in this tissue disappeared and blood cells penetrated into tumor tissue. The tumor cells in the mice injected by free RNase A (Fig. 6b) were without degeneration in the whole tissue and also the vein wall was not damaged.

Fig. 5



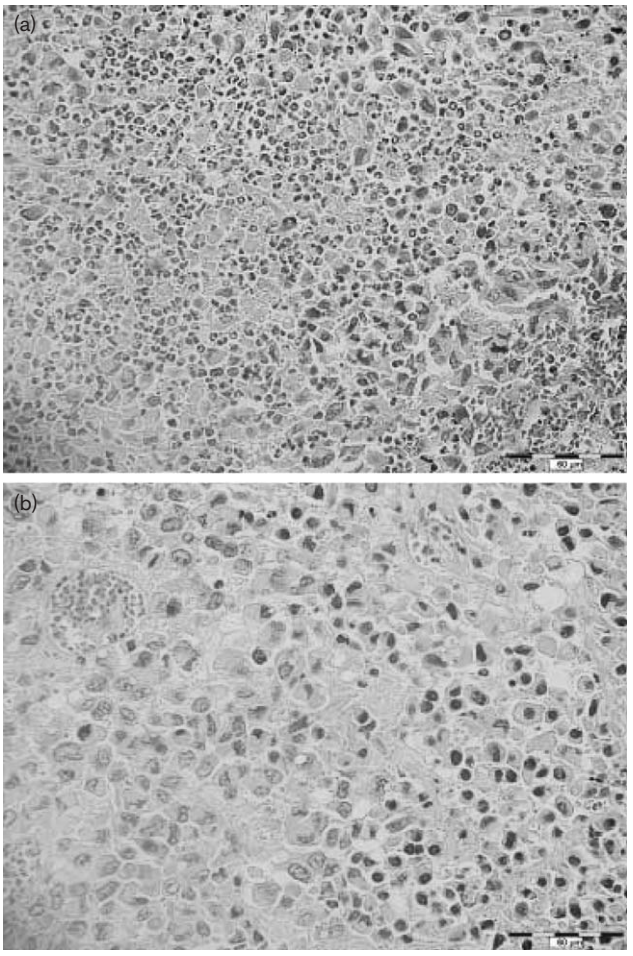
Human melanoma tumor growth in athymic *nu/nu* mice after intraperitoneal (i.p.) injection of wheat leaf ribonuclease (WLN-RNase) and bovine seminal ribonuclease (BS-RNase) conjugated to polyethylene glycol (PEG) (PEG as a control).

Effect of wheat leaf ribonuclease on mice spermatogenesis and on development of early mice embryos

Spermatogenic and early embryonic cells are very effective markers for studying toxic and side-effects of various substances injected into animals. This is the reason why mice testes and early embryos were also used for testing toxicity of ribonucleases. The aspermatogenic

effects of WLN-RNase, RNase A and BS-RNase obtained after intratesticular and intraperitoneal application to mice are given in Table 1 and Fig. 7.

Fig. 6



(a) Effect of wheat leaf ribonuclease (WLN-RNase) + polyethylene glycol (PEG) injected 7 times intraperitoneally into athymic mice bearing human melanoma tumors. Tumor cells degenerated in the tissue, the wall of the vein disappeared and blood cells penetrated into the tissue, and tumor size decreased (see Fig. 5). (b) Effect of free RNase A injected 7 times intraperitoneally into athymic *nu/nu* mice bearing human melanoma tumors. Tumor cells and the vein's wall do not show degeneration, and tumor size was significantly larger in comparison with tumors growing in mice injected with WLN-RNase (see Fig. 5).

In contrast to the application of RNase A, the WLN-RNase and BS-RNase injected intratesticularly resulted in significant reduction of the width of spermatogenic layers and also in a reduced diameter of seminiferous tubules in testes of injected mice (Table 1). The embryotoxic effect of WLN-RNase was equal to that of BS-RNase (Table 2), but lower in comparison with ONC, which was used for comparison as a strong embryotoxic frog ribonuclease. RNase A did not evoke embryotoxicity.

Complex of wheat leaf ribonuclease with its antibodies and its activity on mice spermatogenesis

Antibodies against BS-RNase blocked out the cytotoxic effect of this enzyme *in vitro* [14]. In this study, we tested the effect of WLN-RNase injected with its mice homologous antiserum in experiments *in vivo*. When the WLN-RNase in complex with mice antiserum was injected to the left testes of mice, the marked drop of spermatogenesis appeared (Fig. 7). The same change in aspermatogenic effect was observed when WLN-RNase was injected with normal nonimmune mice serum. It means that WLN-RNase was not blocked in its activity by homologous antibodies *in vivo*.

Immunogenicity and antigen relationship between wheat leaf ribonuclease and some animal ribonucleases

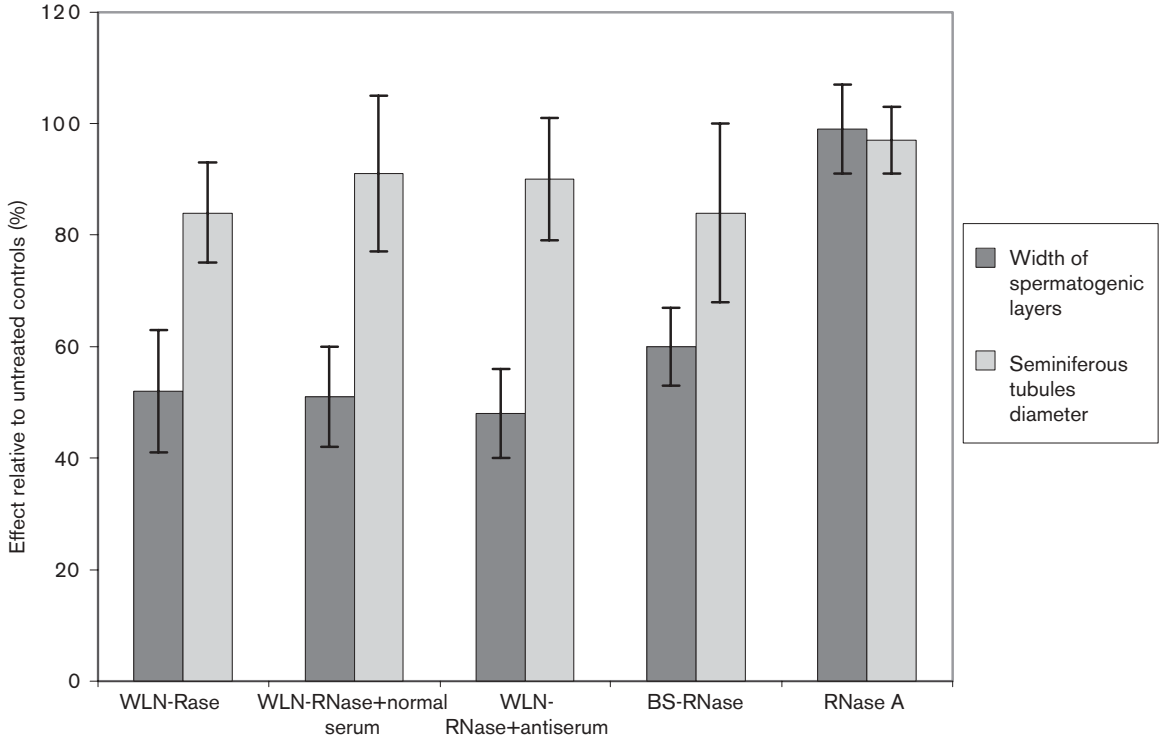
Immunogenicity expressed as production of antibodies in the mice injected with WLN-RNase, RNase A, ONC and HP-RNase appeared to be very low in titers of 0–320 (Table 3). An exception was the production of antibodies against BS-RNase in which titers ranged from 1280 to 2560. The titer of these antibodies is significantly higher in comparison with WLN-RNase. All antibodies against animal RNases reacted with WLN-RNase in the titer 20–80, only with the exception of antibodies against BS-RNase, which reacted with WLN-RNase in the titer 160–320. The same titer (160–320) was noticed with the homologous antibodies against WLN-RNase (Table 3). Due to this reason, it might be supposed that the immunological response of WLN-RNase in mice is lower than that caused by BS-RNase injection. The mutual immunological reactivity (cross-reaction) of WLN-RNase with other animal RNases seems to prove that wheat leaf ribonuclease resembles structurally some animal RNases and vice versa.

Table 1 Aspermatogenic effect in mice after intratesticular injection of WLN-RNase in comparison with the effects due to RNase A and BS-RNase

Substances injected (dose)	No. of mice	Index weight of both testes ± SEM	Width of spermatogenic layers of both testes (μm ± SEM)	Diameter of seminiferous tubules of both testes (μm ± SEM)	Body weight before experiment	Body weight after experiment
PBS	5	92 ± 9	64 ± 11	163 ± 6	22 ± 1	25 ± 1
RNase A	5	87 ± 8	65 ± 5	164 ± 9	22 ± 1	24 ± 1
BS-RNase	5	90 ± 7	32 ± 5**	141 ± 8**	21 ± 2	22 ± 1
WLN-RNase	5	86 ± 6	40 ± 3**a	146 ± 7**	23 ± 1	23 ± 2

PBS, phosphate-buffered saline; BS-RNase, bovine seminal ribonuclease; WLN-RNase, wheat leaf ribonuclease.
[a]Differences between the width of spermatogenic layers of WLN-RNase and BS-RNase (40 ± 3 and 32 ± 5) are significant.
**$P < 0.01$.

Fig. 7



Effect of ribonucleases on spermatogenesis: comparison of free and antibody binding wheat leaf ribonuclease (WLN-RNase), bovine seminal ribonuclease (BS-RNase) and RNase A injected to the left testes of male mice.

Table 2 Development of mice embryos after 72 h incubation with WLN-RNase compared with animal ribonucleases RNase A, BS-RNase and ONC

Enzyme (doses in μg/ml)	No. of embryos	Stage of embryo development			
		Blastocysts	Expanded blastocysts	Total blastocysts	% Blastocysts
Control	12	7	1	8	66
RNase A (100)	10	2	4	6	60
BS-RNase (100)	11	1	0	1	9**
ONC (100)	21	0	0	0	0**
ONC (10)	14	0	0	0	0**
WLN-RNase (100)	12	2	0	2	16**

ONC, onconase; BS-RNase, bovine seminal ribonuclease; WLN-RNase, wheat leaf ribonuclease.
**$P<0.01$.

Discussion

Although plant S-ribonucleases with their gametophytic self-incompatibility functions appeared in the group of RNases involved in RISBASES (ribonucleases with special biological action) [27], the idea that plant ribonucleases can have further functions developed rapidly [4–6]. The plant ribonucleases genetically controlled by the S-like locus (S-like RNases), which participate in correlation with senescence, phosphate starvation, plant diseases, seed development and seed germination, as well as root, fruit and flower development, are physiologically very important. One interesting point is that no plant ribonucleases from these S-RNases and S-like RNases of higher plants were studied for their biological activities on mammalian cells or tissues. Only three groups of authors published the antifungal and antiproliferative activities *in vitro* from the roots of sanchi ginseng (*Panax notoginseng*), ribosome-inactivating protein from the mature seeds of pumpkin (*Cucurbita moschata*) and ribotoxin with ribonuclease activity from the mature seeds of oriental arborvital (*Biota orientalis*) [8–10]. As some S-like plant RNases have a similar molecular weight (26–27 kDa) to BS-RNase, which has several biological activities and some effects on mammalian normal and tumor tissues [1–3,20,21,26–28], we decided to compare these activities of BS-RNase and some other animal ribonucleases with WLN-RNase (27 kDa). The preparation of this enzyme was tested for its RNase activity and

Table 3 Production of antibodies (five mice per group) against WLN-RNase as well as immunological cross-reactions between them

WLN-RNase	WLN-RNase[a]	WLN-RNase	WLN-RNase	WLN-RNase	WLN-RNase
Antibody titers	160–320	20–40	160–320	20–40	40–80
Anti-RNases	Anti-WLN-RNase	Anti-RNase A	Anti-BS-RNase	Anti-ONC	Anti-HPRNase
RNase A	RNase A	RNase A	RNase A	RNase A	RNase A
Antibody titers	40–80	20–80	160–320	40–80	20–40
Anti-RNases	Anti-WLN-RNase	Anti-RNase A	Anti-BS-RNase	Anti-ONC	Anti-HPRNase
BS-RNase	BS-RNase	BS-RNase	BS-RNase[a]	BS-RNase	BS-RNase
Antibody titers	80–160	10 20	1,280–2,560	40	80–160
Anti-RNases	Anti-WLN-RNase	Anti-RNase A	Anti-BS-RNase	Anti-ONC	Anti-HPRNase
ONC	ONC	ONC	ONC	ONC	ONC
Antibody titers	20–40	0–20	80–160	160–320	20–40
Anti-RNases	Anti-WLN-RNase	Anti-RNase A	Anti-BS-RNase	Anti-ONC	Anti-HPRNase
HPRNase	HPRNase	HPRNase	HPRNase	HPRNase	HPRNase
Antibody titers	40–80	40–80	160–320	20–40	160–320
Anti-RNases	Anti-WLN-RNase	Anti-RNase A	Anti-BS-RNase	Anti-ONC	Anti-HPRNase

ONC, onconase; BS-RNase, bovine seminal ribonuclease; WLN-RNase, wheat leaf ribonuclease.
[a]Significant differences in production of antibodies $P<0.01$.

purity, and was found to be well characterized. The presence of the glycosidase activity in 27-kDa WLN-RNase was not proved.

Wheat as an important plant growing in many parts of the world could be suitable for its utilization in some biologic and antitumor studies. The in-vitro inhibition effect of WLN-RNase on ML-2 cell proliferation, however, was negligible compared to that of BS-RNase. On the other hand, human melanoma tumor growing in the athymic mice degenerated after intratumoral administration of WLN-RNase similarly compared with BS-RNase. WLN-RNase after its conjugation with PEG and injected intraperitoneally was cytotoxically more efficient and its antitumor activity balanced to PEG-conjugated BS-RNase. This conjugation was shown to be very important also for the antitumor activity of RNase A, which displayed antitumor activity only when conjugated with PEG or PHPMA (poly[*N*-(-2-hydroxypropyl)methacrylamide]) [22,23,29].

The results presented in our study, and referred to our previous studies, suggest the existence of three positive mechanisms of action caused by PEG and PHMA conjugation to RNases. (1) Both polymers protect RNases with low biological activity against the action of ribonuclease inhibitor and change it into a cytotoxically active substance; (2) the polymer-conjugated RNase persists in the blood stream of mice longer, whereas the free form of enzyme is very quickly eliminated; and (3) the modification of ribonuclease enzyme by a polymer increases its resistance to proteolytic attack [22,23,29]. The smaller immunogenicity of WLN-RNase in comparison with BS-RNase may also be one of the reasons for the stability of this enzyme in the blood stream. Structural similarity between WLN-RNase and animal RNases was proved immunologically by cross-reaction of antibodies against animal RNase with WLN-RNase and cross-reaction of antibodies against WLN-RNase with animal RNases. Immunogenicity of WLN-RNase, which is also characterized by a certain intensity of antibody production, was similar to that of RNase A, ONC and HP-RNase. This immunogenicity, however, was much smaller in comparison with BS-RNase. This observation is surely positive for WLN-RNase administration as the production of antibodies would not be as intensive as the production of antibodies after BS-RNase injections. The complex of WLN-RNase with own antibodies and injected into left testes of mice degenerated the spermatogenic epithelium with the same intensity as WLN-RNase alone, without antibodies or with normal mice serum. This situation proves that antibodies against WLN-RNase do not block the aspermatogenic effect of this plant enzyme. It is possible to suppose that this phenomenon could be the same or similar to other biological activities in mice injected by this ribonuclease. Almost the same aspermatogenesis evoked by injection of BS-RNase complex with antibodies in titers 250 000–500 000 or with BS-RNase alone (without antibodies) supports this possibility [13]. Of course, it would be also possible that the WLN-RNase–antibodies complex is not stable and it is disconnected in the in-vivo setting. The antitumor activity of WLN-RNase observed in these experiments may also be supported by preliminary studies of Mung bean nuclease. This plant enzyme, injected intratumorally or intraperitoneally in its PEG conjugated form into mice bearing melanoma tumor, exerted an even higher antitumor effect than WLN-RNase (Matousek *et al.*, 7th International Meeting on Ribonucleases, 16–20 June 2005, Stará Lesná, Slovak Republic. Abstracts p. 81).

A certain risk for mammals of plant proteins isolated from pollen consists of their allergic effects [30]. The allergic

substances, however, have not been observed yet in plant leaves. Indeed, no mice investigated in our experiments were assigned allergic symptoms after WLN-RNase injections.

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References

1 D'Alessio G, Riordan JF. *Ribonucleases, Structures and Function*. San Diego: Academic Press; 1997.
2 Matousek J. Ribonucleases and their antitumor activity (Review). *Comp Biochem Physiol C* 2001; **129**:175-191.
3 Makarov AA, Ilinskaja ON. Cytotoxic ribonucleses: molecular weapons and their targets. *FEBS Lett* 2003; **540**:15–20.
4 Green JP. The ribonucleases of higher plants. *Annu Rev Plant Physiol Mol Biol* 1994; **45**:421–445.
5 Bariola PA, Green PJ. Plant Ribonucleases. In: D'Allessio G, Riordan JF, eds. *Ribonucleases, Structures and Function*. San Diego: Academic Press; 1997, pp. 163–190.
6 Parry SK, Liu Y, Clarke AE, Newbigin E. S-RNases and others plant extracellular ribonucleases. In: D'Alessio G, Riordan JF, eds. *Ribonucleases, Structures and Function*. San Diego: Academic Press; 1997, pp. 192–208.
7 Dangl JL, Dietrich RA, Thomas H. Senescence and programmed cell death. In: Buchman B, Gruissen W, Jones R, eds. *Biochemistry and Molecular Biology of Plants*. Rockville, MD: Amer. Soc. Plant Physiol.; 2000, pp. 1004–1100.
8 Lam SK, Ng TB. Isolation of a novel thermolabile heterodimeric ribonuclease with antifungal and antiproliferative activities from roots of the Sanchi Ginseng Panax notoginseng. *Biochem Biophys Res Commun* 2001; **285**:419–423.
9 Xia HC, Li F, Li Z, Zhang ZC. Purification and characterization of Moschatin, a novel type I ribosomeinactivating protein from the mature seeds of pumkin (Cucurbita moschata), and preparation of its immunotoxin against human melanoma cells. *Cell Res* 2003; **13**:369–374.
10 Xu H, He WJ, Liu WY. A novel ribotoxin with ribonuclease activity that specifically cleaves a single phophodiester bond in rat 28S ribosomal RNA and inactivates ribosome. *Arch Biochem Biophys* 2004; **427**:30–40.
11 Chang S-C, Gallie DR. RNase activity decreases following a heat shock in wheat leaves and correlates with its posttranslation modification. *Plant Physiol* 1997; **113**:1253–1263.
12 Booker FL. Influence of ozone on ribonuclease activity in wheat (Triticum aestivum) leaves. *Physiol Plant* 2004; **120**:249–255.
13 Dostal J, Matousek J. Purification of aspermatogenic substance (AS) from the bull seminal vesicle fluid. *J Reprod Fertil* 1972; **31**:273–275.
14 Matousek J. Aspermatogenic effect of the bull seminal ribonuclease (BS-RNase) in the presence of anti-BS-RNase antibodies in mice. *Anim Genet* **25**, 1994; 1 (Suppl):45–50.
15 Kim BM, Schultz LW, Raines RT. Variants of ribonuclease inhibitor that resists oxidation. *Protein Sci* 1999; **8**:430–434.
16 Westermeyer R. *Electrophoresis in Practice*. 3rd Ed. Germany: Willey-WCH Weinheim; 2001.
17 Eisental P, Danson MJ. *Enzyme Assays - A Practical Approach*. Oxford University Press, 1993.
18 Harris JM, Zalipsky S. *Poly (ethylene glycol): Chemistry and Biology Application*, ACS Washington D.C. 1997.
19 Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**:248–252.
20 Soucek J, Marinov J, Benes J, Hilgert I, Matousek J, Raines RT. Immunosuppressive activity of bovine seminal ribonuclease and its mode of action. *Immunobiology* 1996; **195**:271–285.
21 Soucek J, Raines RT, Hengg M, Raillard-Yoon S, Benner SA. Structural changes to ribonuclease A and their effects on biological activity. *Comp Biochem Physiol C* 1999; **123**:103–111.
22 Matousek J, Pouckova P, Soucek J, Skvor J. PEG chains increase aspermatogenic and antitumor activity of RNase A and BS-RNase. *J Control Release* 2002; **82**:29–37.
23 Pouckova P, Zadinova M, Houskova D, Strohalm J, Placova D, Spunda M, *et al.* Polymer-conjugated bovine pancreatic and seminal ribonucleases inhibit growth of human tumors in nude mice. *J Control Release* 2004; **95**:83–92.
24 Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J Reprod Fertil* 1989; **86**:679–688.
25 Tahara T, Usuki K, Sato H, Ohashi H, Morita H, Tsumura H, *et al.* A sensitive sandwich ELISA for measuring thrombopoietin in human serum: Serum thrombopoietin levels in healthy volunteers and in patients with haemopoietic disorders. *Br J Haematol* 1996; **93**:783–788.
26 D'Alessio G, Di Donato A, Parente A, Piccoli R. Seminal RNase: a unique member of the ribonuclease superfamily. *Trends Biochem. Sci* 1991; **16**:104–106.
27 Irie M. Structures and function of ribonucleases. *Yakugaku Zasshi* 1997; **117**:561–582.
28 Mastronicola MR, Piccoli R, D'Alessio G. Key extracellular and intracellular steps in the antitumor action of seminal ribonuclease. *Eur J Biochem* 1995; **230**:242–249.
29 Ulbrich K, Strohalm J, Plocova D, Oupicky D, Subr J, Soucek J, *et al.* Poly [N-(hydroxypropyl) methacrylamide] conjugates of bovine seminal ribonuclease. Synthesis, physicochemical and preliminary biological evaluation. *J Bioact Comp Pol* 2000; **15**:4–26.
30 Bufe A, Uhlig U, Sholzen T, Matousek J, Schlaak M, Weber W. A nonspecific, single-stranded nuclease activity with characteristics of a topoisomerase, found in a major grass pollen allergen: Possible biologic significance. *J Biol Chem* 1999; **380**:1009–1016.