

Structural changes to ribonuclease A and their effects on biological activity

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Received 26 May 1998; received in revised form 15 December 1998; accepted 18 December 1998

Abstract

Bovine seminal ribonuclease (BS RNase) displays immunosuppressive and antitumor activities on mammalian cells, whereas bovine pancreatic ribonuclease (RNase A) is not cytotoxic. To learn more about the mechanism of BS RNase cytotoxicity, various mutants and hybrid proteins were prepared. A series of RNase A variants substituted with amino acid residues from BS RNase were prepared. Concerning quaternary structure, a significant impact was achieved in the variant TM (Q28L K31C S32C), which forms a dimer joined covalently by two intersubunit disulfide bonds. This variant is more efficient than RNase A but less active than BS RNase. Introduction of cationic residues at positions 55, 62, and 64 or substitution at positions 111 and 113 enhanced the immunosuppressive activity of RNase A but did not confer its antitumor activity. The substitution at positions 28, 31, 32, 55, 62, 64, 111, and 113 in variant T13 exerted the best immunosuppressive and antitumor effect observed among the round of the RNase A variants. Replacement of the active-site histidine residues H12 and H119 with asparagine led to the loss of both catalytic and biological activities. Five previously prepared hybrid enzymes (SRA 1–5), synthesized by introducing 16 amino acid residues from RNase A into BS RNase, exerted the same immunosuppressive activities as did the wild-type BS RNase. However, the substitution at positions 111, 113, and 115 in variant SRA 5 caused a marked decrease in its antitumor effect, indicating that these residues play an important role in antitumor efficiency. A different mechanism of action of RNases on tumor cells and/or on blastogenic transformed lymphocytes has been assumed. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Bovine seminal ribonuclease; Ribonuclease variants; Structure; Antitumor action; Immunosuppression

1. Introduction

Bovine seminal ribonuclease (BS RNase), a dimeric homolog, differs in 23 amino acid residues from bovine pancreatic ribonuclease (RNase A), the prototype of the ribonuclease superfamily [6,29]. In contrast to RNase A and monomeric BS RNase, dimeric BS RNase displays various biological properties including

aspermatic [23], antitumor [18,21,25,36,40,43], embryotoxic [22] and immunosuppressive [37,39,41,42] activities. Because of these special properties, BS RNase has been ranked among the ribonucleases with a special biological action, denoted RISBASes [6]. Previously, we reported [14,15,38,39] that BS RNase expressed heterologously in *Escherichia coli* [11] is as biologically active as the wild-type BS RNase, isolated and purified from bovine seminal vesicles [9]. This ability of recombinant BS RNase was also reported by others [1,5,8]. These findings proved definitively that wild-type BS RNase itself is an immunosuppressive and antitumor

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agent, regardless of some negligible contaminants that might occur even in highly purified preparations. We have also reported that BS RNase binds very potently to the surface of various tumor cells [24,35] or phytohemagglutinin (PHA)-stimulated lymphocytes [36,41] and that it is situated within less than 1 h in the cell cytosol [39]. Recently, several research groups have tried to elucidate the mode of action of BS RNase and the fate of this enzyme in the cytosolic environment of the target cell [15,19,20,28,44]. The other authors have synthesized various RNase A and BS RNase hybrids, and have tested their biological activity to learn more about the sites and mechanisms of their action [3,8,13–15,19]. Di Donato et al. [8] examined a set of RNase A mutants on a mice fibroblast cell line transformed with SV40 virus. They found a close correlation between the cytotoxic activity of RNase A variants against transformed cell lines and their ability to form a swapped dimer. To elucidate the mechanism of BS RNase cytotoxicity, Kim et al. [13–15] constructed a series of BS RNase variants and examined their cytotoxicity against two human tumor cell lines. The authors suggest that dimeric BS RNase enters cells by adsorptive rather than receptor mediated endocytosis [15]. Benner et al. [3] tried to find out which of 23 amino acid residues that separate seminal RNase and RNase A is responsible for the special biological behaviour. They prepared a series of hybrid proteins by introducing amino acids from seminal RNase into an RNase A background and analyzed their quaternary structure, catalytic activity, and ability to bind and melt duplex DNA [3,30].

Onconase, the most interesting ribonuclease belonging to the RNase A superfamily, was discovered recently [2]. This monomeric RNase isolated from eggs of a frog, *Rana pipiens*, displays a very potent antitumor activity. How onconase kills the tumor cells is not well known but unlike RNase A, onconase is suggested to be bound to the cell surface by a specific receptor and to be resistant to the action of cytosolic ribonuclease inhibitor [45].

Angiogenin (ANG), another member of the RNase A superfamily, belongs also among those ribonucleases with special biological action [33]. ANG promotes the formation of blood vessels in animals, whereas RNase A possesses no angiogenic activity. ANG is a poor ribonuclease but its ribonucleolytic activity is essential for its angiogenic activity. A hybrid protein, in which 13 residues of a divergent surface loop of ANG were substituted for 15 analogous residues of RNase A, resulted in RNase A endowed with angiogenic activity [31]. Previously, we reported on the immunosuppressive activity of two human angiogenin preparations [26].

In the present study, we have assembled an extensive series of mutated ribonucleases and compared their immunosuppressive and antitumor activities in relationship to their structures. Preliminary results on the struc-

tural basis for biological activities of 30 RNase mutants have already been presented [38].

2. Materials and methods

2.1. Ribonucleases and preparation of their hybrids

A study of the isolation and purification of BS RNase from bovine seminal vesicles was published earlier [9]. Recombinant wild-type BS RNase was produced in *E. coli* by using expression vector pLSRI as described [11]. RNase A was obtained from Boehringer (Germany). The preparation of a series of BS RNase mutants was also described in previous publications [13–15]. A series of hybrid proteins was prepared by introducing amino acids from BS RNase into RNase A. Variants TBS, TM, KKT, KR, and GK were prepared as described in Raillard-Yoon's dissertation [30].

Plasmids containing the genes encoding variants of RNase A, A(Q28L K31C S32C), A(Q55K N62K A64T), and A(E111G N113K), were digested with restriction enzyme StyI, yielding two fragments (3989 and 1244 bp, respectively). The large fragment from the first plasmid was ligated with a small fragment of either the plasmid carrying the A(Q55K N62K A64T) variant or the plasmid carrying the A(E111G N113K) variant. A gene encoding the variant A(Q28L K31C S32C Q55K N62K A64T E111G N113K) was prepared from the gene for variant A(Q28L K31C S32C Q55K N62K A64T) and the oligonucleotide encoding the E111G N113K substitutions by the method of Kunkel et al. [17]. The mutant proteins were reconstituted and purified by Ciglic in a Zurich laboratory [3].

2.2. Assay of immunosuppressive activity

The effect of RNases on the proliferation of human lymphocytes stimulated in mixed lymphocyte culture (MLC) was assessed as previously described [39]. Briefly, the lymphocytes were isolated on a Ficol-Paque solution gradient from heparinized blood of healthy persons. The cells of two unrelated individuals were mixed (1:1) and resuspended in RPMI 1640 medium supplemented with heat-inactivated human AB serum and antibiotics. The lymphocyte suspension ($100 \mu\text{l}/10^{-5}$ cells) was pipetted in a microtiter plate type U (produced by A/C NUNC, Denmark) and aliquots of tested RNases (100 μl) at various concentrations were added in three replicate samples. After 6 days of cultivation at 37°C in a humidified atmosphere with CO₂ (5% v/v), the cell culture was pulsed with 24 kBq of ³H-thymidine for 4 h. The incorporated radioactivity in harvested cells was measured by a beta counter (Beckman). The mean values of triplicates expressed as counts per minute (cpm) were calculated and the sup-

pression of DNA synthesis caused by RNase was expressed as the percentage of control cpm values.

2.3. Antiproliferative activity assay

The cytotoxic effect of RNase variants was tested on various stabilized human cell lines derived from hematological malignancies as previously described [40]. We have chosen cell line K 562 derived from human erythroleukemia and cell lines ML-1 and ML-2 derived from acute myeloid leukemia. Briefly, 2×10^5 cells in 0.2 ml of RPMI 1640 medium supplemented with FCS (10% v/v) and antibiotics were cultivated in microtiter plates type FB (A/C NUNC, Denmark) for 2 or 3 days under the same conditions as already mentioned. A selected concentration of RNase was added at the beginning of the experiment to each of three replicate cultures. After 2 or 3 days of incubation, the proliferation of cultured cells was estimated on the basis of ^3H -thymidine incorporation into newly synthesized cell DNA as before.

3. Results

3.1. Immunosuppressive and antitumor activity of RNase A variants

A series of RNase A variants was synthesized by replacing several regions in the protein where RNase A differs from biologically active BS RNase [3,30]. The antiproliferative effect of these RNase variants on

MLC-stimulated lymphocytes or two tumor cell lines is summarized in Table 1. As the first focal point, we chose a region that lies at the contact site between the subunits of dimeric BS RNase. The dimeric variant of RNase A substituted as Q28L K31C and S32C, and designated TM, exerted remarkable immunosuppressive and antitumor activity. Although considerably more active than RNase A, variant TM is not as active as BS RNase. This result may support the previous assumption on the importance of the dimeric form for biological efficiency [5,7,8,15]. Interestingly, the dimeric variant (K31C S32C) was not immunosuppressive (unpublished data), suggesting that the dimeric form is not sufficient for immunosuppressive activity.

The second step involved replacing seven amino acid residues (Q55K N62K A64T Y76K S80R E111G and N113K) in RNase A by the residues of BS RNase, recreating the cationic surface. The resulting variant was designated TBS. The immunosuppressive and antitumor activity of this monomeric compound increased slightly compared with that of RNase A. Introduction of three segments of residues forming the total cationic surface of BS RNase /55, 62, 64/, /76, 80/ and /111, 113/ into the RNase A molecule (Fig. 1) was used to test which of these segments contribute to the biological activity of BS RNase. Results in Table 1 demonstrate that introduction of residues 55, 62, and 64 or residues 111 and 113 markedly increases the immunosuppressive activity but has little effect on the antitumor activity of RNase A, whereas introduction of cationic side chains at positions 76 and 80 displays no immunosuppressive or antitumor activities. These results were very surpris-

Table 1
Immunosuppressive and antitumor activity of RNase variants^a

Symbol of RNase	Replaced AA from BS RNase	Form	IC ₅₀ (μg ml ⁻¹) ^b			Immunosuppression	Antitumor activity
			MLC	K 562	ML-1		
A-wild		Mono	200	N.I.	N.I.	+	0
A- <i>E. coli</i>		Mono	80	N.I.	N.I.	+	0
TM	28, 31, 32	Di	16	90	200	+++	+
TBS	Total basic surface	Mono	90	165	200	+	+
KKT	55, 62, 64	Mono	10	N.I.	N.I.	+++	0
KR	76, 80	Mono	N.I.	N.I.	N.I.	0	0
GK	111, 113	Mono	20	N.I.	N.I.	++	0
H12	H12N in TBS	Mono	N.I.	N.I.	N.I.	0	0
DIT	31, 32, 38, 111	Di	32	40	200	++	++
MT	38, 111	Mono	110	180	N.I.	+	±
KMQGK	34, 35, 37, 39	Mono	N.I.	N.I.	N.I.	0	0
Q III	55, 111, 113	Mono	150	200	N.I.	+	±
T1	28, 31, 32, 55, 62, 64	Di	40	200	200	++	+
T3	28, 31, 32, 111, 113	Di	30	40	35	++	+++
T13	28, 31, 32, 55, 62, 64, 111, 113	Di	30	30	10	++	+++
BS-wild		Di	8	11	45	+++	+++
BS- <i>E. coli</i>		Di	5	5	14	+++	+++

^a Mono, monomer; di, dimer; N.I., no inhibition; 0, no effect; + to +++, potency of the biological activity.

^b IC₅₀, concentration of RNase halving the ^3H -thymidine incorporation into newly synthesized cell DNA.

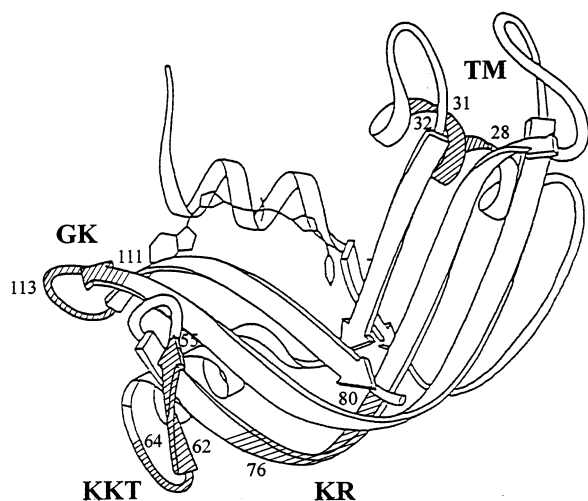


Fig. 1. RNase A molecule with 23 amino acid positions differing from BS RNase. The dashed regions represent four variants where numbered amino acid residues were substituted with those from BS RNase.

ing because both monomeric variants KKT (Q55K N62K A64T) and GK (E111G N113K) proved to be as immunosuppressive as dimeric variant TM (Q28L K31C S32C). In contrast, variant KR (Y76K S80R) displays no immunosuppressivity, appearing to inhibit the immunosuppressive activity conferred by basic substitutions at other positions in the protein.

Replacement of histidine with asparagine H12N or H119N destroyed in RNase A variants both the cata-

lytic and/or the cytotoxic activities to tumor cells or MLC-stimulated lymphocytes (Table 1). This result is consistent with that of H119D BS RNase [13] as shown in Table 2.

Residue 38 was found to confer upon RNase A substantial catalytic activity against double-stranded RNA, and an improved ability to bind to duplex DNA. DIT, a variant charged at this position and with remarkably increased catalytic activity against duplex RNA compared with RNase A, was constructed by making the substitutions K31C, S32C, D38G, and E111G. This dimeric variant displays potentially increased immunosuppressive and antitumor activities compared with those of RNase A, but this increase does not have the potent effect of BS RNase and also does not parallel the very strong increase in catalytic activity. The properties of another monomeric variant, MT (D38G, E111G), are better pronounced than those of RNase A but less pronounced than those of DIT (Table 1). The dimeric form is again more responsible for the biological effect. However, these results suggest that biological activity is not a simple function of enzymatic activity.

Finally a round of hybrid constructions were done to learn whether the substitution of residues from the segmented cationic surface of BS RNase (variants KKT and GK) combined with the dimeric form of RNase A (variant TM) may yield a more efficient variant. Thus three dimeric variants were prepared, T1, T3 and T13, substituted at positions (28, 31, 32, 55, 62, 64),

Table 2
Immunosuppressive and antitumor effect of BS RNase mutants^a

Symbol of RNase	Replaced AA from RNase A ^c	Form	IC ₅₀ (μg ml ⁻¹) ^b			Immunosuppression	Antitumor activity
			MLC	K 562	ML-1		
BS-wild		Di	10	12	50	+++	+++
BS- <i>E. coli</i>		Di	20	20	50	+++	+++
M × M		Di	8	10	25	+++	+++
M = M		Di	10	12	100	+++	++
C31S	31	Di	25	92	>200	+++	+
C32S	32	Di	12	12	180	+++	++
SRA1	16, 17, 19, 20	Di	10	21	100	+++	+++
SRA2	35, 37, 38, 39	Di	10	35	80	+++	+++
SRA3	62, 64	Di	10	25	70	+++	+++
SRA4	80, 102, 103	Di	10	20	55	+++	+++
SRA5	111, 113, 115	Di	21	200	N.I.	+++	+
H119	H119D	Di	N.I.	N.I.	N.I.	0	0
M'-B-M'	Nonred C31S	Di	8	32	200	+++	+++
M	BS- <i>E. coli</i>	Mono	70	N.I.	N.I.	++	0
M'-B	C31S	Mono	32	N.I.	N.I.	++	0
MCM	-CHCO on C32	Mono	200	N.I.	N.I.	+	0
Rnase A		Mono	>200	N.I.	N.I.	+	0
CT	C31K, C32S	Mono	200	N.I.	N.I.	+	0
B38	31, 32, 38	Mono	120	N.I.	N.I.	+	0

^a Mono, monomer; di, dimer; N.I., no inhibition; 0, no effect; + to +++, potency of the biological activity.

^b IC₅₀, concentration of RNase halving the ³H-thymidine incorporation into newly synthesized cell DNA.

^c AA, amino acids.

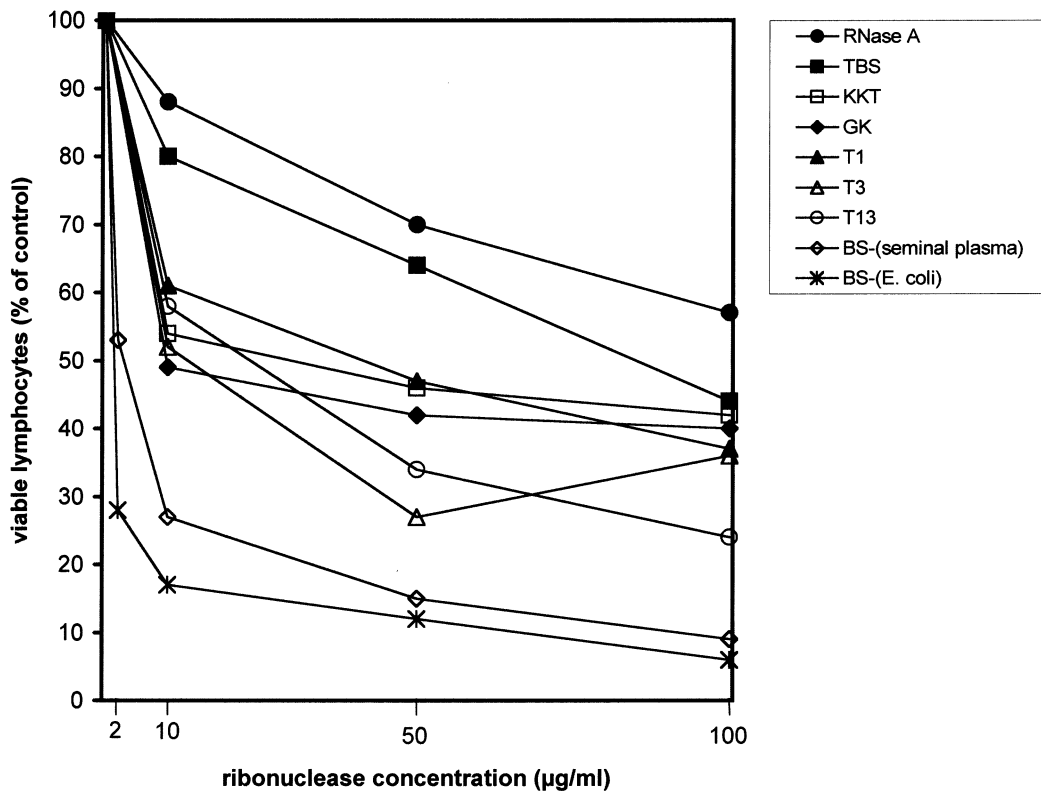


Fig. 2. Effect of RNase A variants and BS RNase on the proliferation of MLC-stimulated human lymphocytes in a 6-day culture. Proliferation was evaluated by the incorporation of [6-³H]thymidine into cellular DNA. Each point is the mean of three replicates from two experiments and is reported as a percentage of a control lacking RNase.

(28, 31, 32, 111, 113) and (28, 31, 32, 55, 62, 64, 111, 113), respectively. The immunosuppressive activity of all these combined variants does not differ considerably from that displayed by the dimeric variant TM (28, 31, 32) or that of monomers KKT (55, 62, 64) and GK (111, 113) (Fig. 2). However, these variants differ remarkably in their antitumor effects. The negligible antitumor effects of KKT and GK was enhanced in variant T1 and even more enhanced in variant T3. The best antitumor activity was gained with combined variant T13 (Fig. 3). This result shows that the introduction of residues 55, 62, 64, 111 and 113 from BS RNase into RNase A leads to an enhancement of antitumor activity, besides the effect of residues 28, 31 and 32 forming the contact site between the two subunits. The enhancing effect of residues 111 and 113 on antitumor activity of variant T3 is consistent with the results reported earlier [15], in which BS RNase lost its antitumor activity after the introduction of residues 111, 113 and 115 from RNase A (variant SRA 5 in Table 2).

3.2. Immunosuppressive and antitumor activity of BS RNase mutants

Furthermore, we wanted to compare our results on RNase A variants and those gained on BS RNase variants. Previously, we constructed a series of the

various hybrid enzymes by introducing amino acid residues from RNase A into a seminal BS RNase molecule and we tested their immunosuppressive effect and their antiproliferative activity [13–15]. The effects of these RNase hybrids on MLC and/or two human tumor cell lines (K 562 and ML-1), are summarized in Table 2. All dimeric mutants exert both immunosuppressive and antitumor activities comparable with the effect of a wild-type BS RNase, whereas all monomers are significantly less immunosuppressive and possess no antitumor activity. All tested BS RNase variants are catalytically active with the exception of the H119D preparation, in which His119, the active-site general acid, is changed to an aspartic acid [13]. This dimeric preparation displays no catalytic and biological activity.

In dimeric mutants of BS RNase, the subunits are cross-linked by two disulfide bonds between Cys-31 of one subunit and Cys-32 of the other subunit. These cross-linked dimers exist in two quaternary forms, designated as $M \times M$ and $M = M$ [29]. The authors have suggested that the two quaternary forms may differ in their enzymatic and biological properties. As we reported [14], the cytostatic activity of our purified $M \times M$ tested on ML-1 line is greater than that of purified $M = M$ (Table 2).

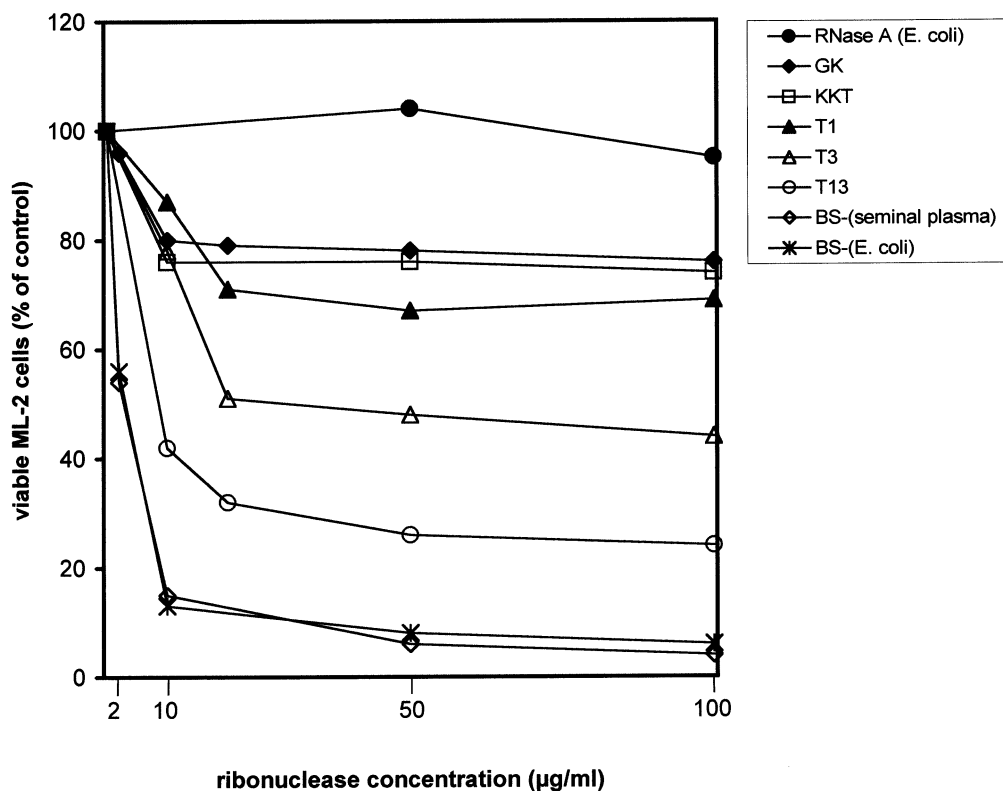


Fig. 3. Effect of RNase A variants and BS RNase on the proliferation of ML-2 leukemia cells in a 48 h-culture. Proliferation was evaluated under the same conditions as mentioned in Fig. 2. Each point is a mean of three replicates and is reported as a percentage of a control lacking RNase. The experiments carried on with K-562 or HUT lines showed the same picture of an inhibitory effect.

Replacing Cys-31 or Cys-32 with a serine residue did not compromise the enzymatic activity of dimeric BS RNase, but reduced both the fraction of $M \times M$ at equilibrium and the cytotoxicity. We prepared a non-reducible covalent dimer ($M'-B-M'$) of C31S BS RNase by using a thiol-specific cross-linking reagent, dibromobimane [12]. This nonreducible dimer C31S BS RNase is unlikely to be in the $M \times M$ form to any significant extent because the $M \times M$ form of C31S BS RNase itself is relatively unstable and because its two subunits are now separated by a bimane group. Yet, this semisynthetic enzyme has cytotoxic activities comparable with those of a wild-type BS RNase. Thus, we have enhanced markedly the cytotoxicity of C31S BS RNase simply by changing the atoms that cross-link the two subunits. This result demonstrates that the domain swapping of the $M \times M$ form is not per se responsible for the cytotoxicity of BS RNase.

4. Discussion

Results reported here define some sequence determinants for immunosuppressive and antitumor activities within the RNase A family. We have tried to determine which of the 23 amino acid substitutions that separate seminal RNase and pancreatic RNase A confer the

distinctive biological behavior on the seminal homolog. The goal was to correlate amino acid substitutions and biological activities with the physical and catalytic differences that result from these substitutions [38].

Bovine seminal ribonuclease is the only known dimeric ribonuclease among the RNase A superfamily. Referring to the numerous studies [5,8,14,18,43], the dimeric form was shown to be essential for the antitumor effect. However, several recent papers [4,27,26,38,45] have shown that cytotoxicity of an RNase is not associated with its dimeric form and that also monomeric RNases may display cytotoxicity against various mammalian cells. Our data in Tables 1 and 2 are consistent with these findings, showing that several monomeric RNases display no antitumor activity but exert remarkable immunosuppressive effect.

The residues 12 and 119, both containing histidines, are essential for catalytic activity against all substrates. Replacement of H119 in dimeric BS RNase with aspartic acid leads to the loss of the catalytic and also immunosuppressive and antitumor activities [13]. Similar effects were also observed with RNase A variants TBS and TM in which histidines at positions 12 or 119 were replaced with asparagine. These variants lost both the catalytic and biological activities (Table 1). Accordingly, the dimeric form in BS RNase is not sufficient for immunosuppressive and/or antitumor action when his-

tidines essential for the catalytic activity are replaced by any other amino acid. Catalytic efficiency was also found to be essential in other biologically active ribonucleases, such as angiogenin [32] and onconase [2].

Sixteen amino acid residues substituted from RNase A to BS RNase in the mutants designated SRA 1–5 had very little effect on the immunosuppressive activity of these five mutants (see Table 2). However, the substitution at positions 111, 113 and 115 position in SRA 5 caused a remarkable decrease in antitumor effect. This result is consistent with that demonstrated in Table 1, where substitution of residues 111 and 113 from BS RNase to RNase A increases the antitumor activity of variants T3 and T13. Thus, the residues G111 and K113 are important for the antitumor potency of BS RNase. The remaining residues substituted in SRA 1–4 (at positions 16, 17, 19, 20, 35, 37, 38, 39, 62, 64, 80, 102 and 103) do not seem to be especially important for the biological effectivity.

DiDonato et al. [8] reported a close correlation between the ability of an RNase variant to exert an antitumor activity and its ability to form a swapped dimer. This report suggests that a dimer in which no swap occurs would be devoid of antitumor activity. However, we prepared a nonreducible covalent dimer ($M'-B-M'$) that is unlikely to be in the form in which the two subunits exchange their N-terminal segments, because its two subunits are separated by a bimeane group. This semisynthetic enzyme, which does not swap its S-peptide chains, has cytotoxic activities comparable with those of wild-type BS RNase. Previously, we found that $M'-B-M'$ is resistant to inhibition by a ribonuclease inhibitor [15]. Thus, we assume that retaining dimeric form in a reducible environment and the resistance to inhibitory effects of a ribonuclease inhibitor (RI) is very important for the biological activity of BS RNase variants. The results demonstrated in Table 1 show that neither the dimeric form nor S-peptide swap are necessary for immunosuppressive activity. For example, variant TM (Q28L K31C S32C), which forms a dimer, is immunosuppressive. However, variant KKT (Q55K N62K A64T) displays similar immunosuppressive activity, even though it does not form a dimer. Furthermore, the RNase A variant (Q28L K31C S32C Q55K N62K A64T E111G N113K), which exerts remarkable immunosuppressive and antitumor activities, although a dimer, swaps its S-peptide in fewer than 20% of the molecules [10].

Several reports demonstrate that the maintenance of the catalytic activity is essential for the biological activity of various RNases [13,32,44]. Some variants prepared in the Zurich laboratory have increased ability to interact with double-stranded nucleic acids [3]. Thus, the variants MT (D38G E111G) and DIT (K31S S32C D38G E111G), with several-fold higher catalytic activities against duplex RNA when compared with RNase

A, have also increased immunosuppressive and antitumor activities (Table 1). However, this increase is not in a correlation with the nearly 30-fold increased catalytic activity. There are other variants with lower catalytic activity against duplex RNA, such as T3 (Q28L K31C S32C E111G N113K) and T13 (Q28L K31C S32C Q55K N62K A64T E111G N113K), that exert higher immunosuppressive and antitumor activities compared with the variants MT and DIT. As mentioned, the monomeric derivatives exert no antitumor effect even though they are catalytically active. These results suggest that biological activity is not a simple function of enzymatic activity.

Murthy and Sirdeshmukh [28] reported that monomeric seminal RNase is inhibited by a RI, while dimeric seminal RNase is not, presumably because the inhibitor makes contact with the RNase in a region of the protein that also forms the dimer contact site [16]. The effect of RI on two quarternary forms of BS RNase showed that RI has a dramatic, differential effect when assays are performed under reducing conditions. These conditions, which are essential for full activity of the RI and are typical for its cytosolic localization, promote monomerization of the $M=M$ form, while the $M \times M$ form remains dimeric. Since cytosolic RI binds tightly to monomeric but not dimeric BS RNase and only $M \times M$ or $M'-B-M'$ forms can resist the reducing environment of cytosol, we propose that the cytotoxic activity of these two mutants arises from the ability to remain dimeric in the cytosol and thus to evade the inhibitory effect of RI. As is shown in Table 2, both these mutants of BS RNase exerted the same cytotoxicity as the wild type. However, two monomeric ribonucleases, onconase [2,44] and angiogenin [26,32], display remarkable cytotoxicity. The immunosuppressive activity of several RNase A variants shown in Table 1 and the aspermatogenic activity of two carboxylated monomers of BS RNase, MCM31 and MCM32 [27], indicate that the dimeric form is not a requirement for ribonuclease cytotoxicity and that the key to ribonuclease biological activity appears to be the evasion of cellular RI [15,27]. This finding was confirmed recently by further publication concerning RNase A variants with potent cytotoxic activity [19]. The authors prepared RNase A variants in which amino acid residues (Asp-38, Gly-88, and Ala-109) that form multiple contacts with RI, were replaced with arginine. They demonstrated that replacing of Gly-88 with arginine or aspartate is sufficient to yield ribonucleases (G88D and G88R) with a decreased affinity for RI and a markedly increased cytotoxicity for tumor cells. Based on these results, we conclude that ribonucleases that retained catalytic activity in the presence of RI are potential cytotoxins.

Immunosuppressive and antitumor effects have been tested on two different models, i.e. MLC-stimulated

human lymphocytes and various human tumor cell lines. It is well known that BS RNase binds very quickly to the cell surface of the PHA-stimulated lymphocytes and/or various tumor cells [20,24,35,39]. The mechanism of antitumor action of BS RNase has already been studied by several authors [1,5,15,18–20,43,44]. They studied the effects of the modified BS RNase structure, the mode of binding on a target cell, internalization by malignant cell and RNA degrading step in this mechanism, respectively. Two of these papers [18,20] have shown that virus-infected fibroblasts are dramatically sensitive to low doses of BS RNase. The essential prerequisite for this antitumor action is a functional catalytic center, which suggests an intracellular RNA degradation. Interestingly, BS RNase is bound and internalized also by corresponding and non-malignant cells, but no effect is detectable on RNase stability or protein synthesis in these cells. The authors assume a different intracellular management in normal cells of the cytotoxic protein [20].

Thus far, it is not clear by which mechanism BS RNase acts as an immunosuppressive agent. Tamburini et al. [42] demonstrated a drastic inhibition of expression of IL-2-alpha chain receptor on OKT3-stimulated lymphocytes, whereas the secretion of IL-2 by T cells was not affected. To elucidate this problem, we studied the effect of BS RNase on mitogen- or MLC-stimulated T and B lymphocytes in culture and we have found a strong inhibitory effect on expression of three activation antigens, i.e. CD25, CD38 and CD71, respectively [39]. We assume that the drastic inhibition of expression of IL-2-alpha chain receptor may induce apoptosis of the proliferating cells. Recently, Bruschke et al. [4] reported that a glycoprotein designated E^{rns}, which is secreted from a pestivirus into the extracellular environment, displays a ribonuclease activity. Since pestivirus infection induces leucopenia, they investigated the immunosuppressive properties of E^{rns} in vitro. The glycoprotein totally inhibited mitogen-induced proliferation of lymphocytes of various species but was not cytotoxic for normal epithelial cells. E^{rns} also strongly inhibited protein synthesis of the treated lymphocytes. Finally, the authors detected an apoptotic process in the cells incubated with E^{rns}. This report is consistent with our preliminary experiments showing induction of an apoptotic process by BS RNase in Con A-stimulated human lymphocytes [34]. Our previous in vitro experiments showed that tumor cells incubated with BS RNase are mostly destroyed, whereas the proliferation of stimulated lymphocytes is inhibited without cell membrane damage. This difference in membrane resistance might support our presumption that a tumor cell may react with RNases differently to a transformed lymphocyte.

Acknowledgements

This work was supported by grants No. 523/96/1738 and No. 307/96/K226 from Grant Agency of the Czech Republic. We are indebted to Gabriela Lindnerová for her excellent technical assistance.

References

- [1] Adinolfi BS, Cafaro V, D'Alessio G, Didonato A. Full antitumor action of recombinant seminal ribonuclease depends on the removal of its N-terminal methionine. *Biochem Biophys Res Commun* 1995;213:525–32.
- [2] Ardelit W, Mikulski SM, Shogen K. Amino acid sequence of an anti-tumor protein from *Rana pipiens* oocytes and early embryos. *J Biol Chem* 1991;266:245–51.
- [3] Benner SA, Ciglic MI, Haugg M, Jermann TM, Opitz JG, Raillard-Yoon SA, Souček J, Stackhouse J, Trabesinger-Rüf N, Trautwein K, Zankel TR. Evolutionary reconstructions in the ribonuclease family. In: D'Alessio G, Riordan JF, editors. *Ribonucleases structures and functions*. New York: Academic Press, 1997:213–43.
- [4] Bruschke ChJM, Hulst MM, Moormann RJM, Van Rijn PA, Van Oirschot JT. Glycoprotein E^{rns} of pestiviruses induces apoptosis in lymphocytes of several species. *J Virol* 1997;71:6692–6.
- [5] Cafaro V, Delorenzo C, Piccoli R, Bracale A, Mastronicola MR, Didonato A, D'Alessio G. The antitumor action of seminal ribonuclease and its quaternary conformations. *FEBS Lett* 1995;359:31–4.
- [6] D'Alessio G, DiDonato A, Parente A, Piccoli R. Seminal RNase: a unique member of the ribonuclease superfamily. *Trends Biochem Sci* 1991;16:106–8.
- [7] DiDonato A, D'Alessio G. Interchain disulfide bridges in ribonuclease BS-1. *Biochem Biophys Res Commun* 1973;55:919–28.
- [8] DiDonato A, Cafaro V, Romeo I, D'Alessio G. Hints on the evolutionary design of a dimeric RNase with special bioactions. *Protein Sci* 1995;4(8):1470–7.
- [9] Dostál J, Matoušek J. Isolation and some chemical properties of aspermatogenic substance from bull seminal vesicle fluid. *J Reprod Fert* 1973;33:263–74.
- [10] Jermann TM, Opitz JG, Stackhouse J, Benner SA. Reconstructing the evolutionary history of the artiodactyl ribonuclease superfamily. *Nature* 1995;374:57–9.
- [11] Kim JS, Raines RT. Bovine seminal ribonuclease produced from a synthetic gene. *J Biol Chem* 1993;268:17392–6.
- [12] Kim JS, Raines RT. Dibromobimane as a fluorescent crosslinking reagent. *Anal Biochem* 1995;225:174–6.
- [13] Kim JS, Souček J, Matoušek J, Raines RT. Catalytic activity of bovine seminal ribonuclease is essential for its immunosuppressive and other biological activities. *Biochem J* 1995;308:547–50.
- [14] Kim JS, Souček J, Matoušek J, Raines RT. Structural basis for the biological activities of bovine seminal ribonuclease. *J Biol Chem* 1995;270:10525–30.
- [15] Kim JS, Souček J, Matoušek J, Raines RT. Mechanism of ribonuclease cytotoxicity. *J Biol Chem* 1995;270:31097–102.
- [16] Kobe B, Deisenhofer J. A structural basis of the interaction between leucine-rich repeats and protein ligands. *Nature* 1995;374:183–6.
- [17] Kunkel TA, Roberts JD, Zakour RA. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* 1987;154:367–82.
- [18] Laccetti P, Portella G, Mastronicola MR, Russo A, Piccoli R, D'Alessio G, Vecchio G. In vivo and in vitro growth-inhibitory

- effect of bovine seminal ribonuclease on a system of rat thyroid epithelial transformed cells and tumours. *Cancer Res* 1992; 52:4582–6.
- [19] Leland PA, Schultz LW, Kim BM, Raines RT. Ribonuclease A variants with potent cytotoxic activity. *Proc Natl Acad Sci USA* 1998;95:10407–12.
- [20] Mastronicola MR, Piccoli R, D'Alessio G. Key extracellular and intracellular steps in the antitumor action of seminal ribonuclease. *Eur J Biochem* 1995;230:242–9.
- [21] Matoušek J. The effect of bovine seminal ribonuclease on cells of Crocker tumor in mice. *Experientia* 1973;29:858–9.
- [22] Matoušek J. Embryotoxic effect of bull seminal ribonuclease and tissue absorption studies in rats. *J Reprod Fert* 1975;43:171–4.
- [23] Matoušek J. Aspermatogenic effect of the bull seminal ribonuclease (BS RNase) in the presence of anti-BS RNase antibodies in mice. *Anim Gen* 1994;25(Suppl. 1):45–50.
- [24] Matoušek J, Fortýn K. Binding of bull seminal ribonuclease (AS RNase) to human tumour tissues. *Folia Biol (Praha)* 1977; 23:366–8.
- [25] Matoušek J, Staněk R. Action of bull seminal vesicle ribonuclease on mouse leukemic cells BP-8 and EL-4. *Folia Biol (Praha)* 1977;23:56–65.
- [26] Matoušek J, Souček J, Říha J, Zankel TR, Benner SA. Immunosuppressive activity of angiogenin in comparison with bovine seminal ribonuclease and pancreatic ribonuclease. *Comp Biochem Physiol B Comp Biochem* 1995;112:235–41.
- [27] Matoušek J, Kim JS, Souček J, Říha J, Ribo M, Leland PA, Raines RT. Ribonucleases endowed with specific toxicity for spermatogenic layers. *Comp Biochem Physiol B Comp Biochem* 1997;118:881–8.
- [28] Murthy BS, Sirdeshmukh R. Sensitivity of monomeric and dimeric forms of bovine seminal ribonuclease to human placental ribonuclease inhibitor. *Biochem J* 1992;281:343–8.
- [29] Piccoli R, Tamburrini M, Piccialli G, DiDonato A, Parente A, D'Alessio G. The dual-mode quaternary structure of seminal Rnase. *Proc Natl Acad Sci USA* 1992;89:1870–4.
- [30] Raillard-Yoon SA. PhD Thesis No. 10261. Zurich: ETH, 1993.
- [31] Raines RT, Toscano MP, Nierengarten DM, Hoi Ha J, Auerbach R. Replacing a surface loop endows ribonuclease A with angiogenic activity. *J Biol Chem* 1995;270:17180–4.
- [32] Shapiro R, Vallee BL. Site-directed mutagenesis of histidine-13 and histidine-114 of human angiogenesis. *Biochemistry* 1989;28: 7401–8.
- [33] Shapiro R, Strydom DJ, Olson K, Vallee BL. Isolation of angiogenin from normal human plasma. *Biochemistry* 1987;26: 5141–6.
- [34] Souček J, Marinov I. An immunosuppressive agent—bovine seminal ribonuclease induces apoptosis in human lymphocytes. In Abstracts of the 10th International Congress of Immunology. New Delhi, 1998:497.
- [35] Souček J, Matoušek J. The binding of bull seminal ribonuclease and its carboxymethylated derivative to human leukaemic cells. *Folia Biol (Praha)* 1979;25:142–4.
- [36] Souček J, Matoušek J, Chudomel V, Lindnerová G. Inhibitory effect of bovine seminal ribonuclease on activated lymphocytes lymphoblastoid cell lines in vitro. *Folia Biol (Praha)* 1981;27: 334–45.
- [37] Souček J, Chudomel V, Potmešilová I, Novák JT. Effect of ribonucleases on cell mediated lympholysis reaction and on GM–CFC colonies in bone marrow culture. *Nat Immun Cell Growth Regul* 1986;5:250–8.
- [38] Souček J, Kim JS, Raillard-Yoon SA, Haugg M, Zankel TR, Raines RT, Benner SA. Structural basis for the immunosuppressive and antitumor activities of ribonucleases mutants. In: Proceedings of the Fourth International Meeting on Ribonucleases. Groningen (The Netherlands), 1996:Abstract S5P5.
- [39] Souček J, Marinov I, Beneš J, Hilgert I, Matoušek J, Raines RT. Immunosuppressive activity of bovine seminal ribonuclease and its mode of action. *Immunobiology* 1996;195:271–85.
- [40] Souček J, Poučková P, Matoušek J, Dostál J, Zadínová M. Antitumor action of bovine seminal ribonuclease. *Neoplasma* 1996;43:335–40.
- [41] Staněk R, Činát J, Matoušek J. The effect of bovine pancreatic ribonuclease [A RNase] and bull seminal ribonuclease [AS RNase] on human lymphocytes undergoing blastic transformation. *Folia Biol (Praha)* 1978;24:111–7.
- [42] Tamburrini M, Scala G, Verde C, Ruocco MR, Parente A, Venuta S, D'Alessio G. Immunosuppressive activity of bovine seminal RNase on T-cell proliferation. *Eur J Biochem* 1990; 190:145–8.
- [43] Vescia S, Tramontano D, Augusti-Tocco G, D'Alessio G. In vitro studies on selective inhibition of tumor cell growth by seminal ribonuclease. *Cancer Res* 1980;40:3740–7.
- [44] Wu YN, Saxena SK, Ardelt W, Gadina M, Mikulski SM, De Lorenzo C, D'Alessio G, Youle RJ. A study of the intracellular routing of cytotoxic ribonucleases. *J Biol Chem* 1995;270:17476–81.
- [45] Youle RJ, Newton D, Wu YN, Gadina M, Rybak SM. Cytotoxic ribonucleases and chimeras in cancer therapy. *Crit Ref Ther Drug Carrier Syst* 1993;10:1–28.