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Toxicity of an Antitumor Ribonuclease to Purkinje Neurons

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Purkinje cell toxicity is one of the characteristic features of the Gordon phenomenon, a syndrome manifested by ataxia, muscular rigidity, paralysis, and tremor that may lead to death (Gordon, 1933). Two members of the RNase superfamily found in humans, EDN (eosinophil-derived neurotoxin) and ECP (eosinophil cationic protein), cause the Gordon phenomenon when injected intraventricularly into guinea pigs or rabbits. We have found that another member of the RNase superfamily, an antitumor protein called onconase, isolated from *Rana pipiens* oocytes and early embryos, will also cause the Gordon phenomenon when injected into the cerebrospinal fluid of guinea pigs at a dose similar to that of EDN (LD_{50}, 3–4 μg). Neurologic abnormalities of onconase-treated animals were indistinguishable from those of EDN-treated animals, and histology showed dramatic Purkinje cell loss in the brains of onconase-treated animals. The neurotoxic activity of onconase correlates with ribonuclease activity. Onconase modified by iodoacetic acid to eliminate 70% and 98% of the ribonuclease activity of the native enzyme displays a similar decrease in ability to cause the Gordon phenomenon. In contrast, the homologous bovine pancreatic RNase A injected intraventricularly at a dose 5000 times greater than the LD_{50} dose of EDN or onconase is not toxic and does not cause the Gordon phenomenon. A comparison of the RNase activities of EDN, onconase, and bovine pancreatic RNase A using three pancreatic RNA substrates demonstrates that onconase is orders of magnitude less active enzymatically than EDN and RNase A. Thus, another member of the RNase superfamily in addition to EDN and ECP can cause the Gordon phenomenon. Ribonuclease activity of onconase appears essential for onconase-mediated neurotoxicity. However, substantial differences in neurotoxicity observed among some homologous ribonucleases cannot be due to their different enzymatic activities; other features of the enzymes are considered.

[Key words: eosinophil-derived neurotoxin (EDN), onconase, ribonucleases, RNase A, Purkinje cells, Gordon phenomenon]

In 1933, Gordon reported that the intracerebral injection of suspensions from human lymphadenomas into rabbits or guinea pigs caused muscular rigidity, incoordination, ataxia, spastic paralysis, and weight loss usually leading to death within 3 d to 1 month. This syndrome became known as the Gordon phenomenon. The pathogenic agent (Turner et al., 1938) in the lymphadenoma extract responsible for the destruction of cerebellar Purkinje cells (Kelser and King, 1936; King, 1939) was identified to be the eosinophil. Two proteins isolated from eosinophils, EDN (eosinophil-derived neurotoxin) and ECP (eosinophil cationic protein), have since been shown to be the active components (Durack et al., 1981; Fredens et al., 1982). EDN and ECP are 67% homologous to one another (Barker et al., 1989; Hamann et al., 1989; Rosenberg et al., 1989a,b) and both are members of the ribonuclease A superfamily (Gleich et al., 1986; Gullberg et al., 1986; Slifman et al., 1986), with approximately 35% identity to the human pancreatic RNase (Barker et al., 1989; Hamann et al., 1989; Rosenberg et al., 1989a,b). The ribonuclease activity of EDN is similar to that of RNase A (Slifman et al., 1986), whereas ECP is approximately 100 times less enzymatically active than EDN (Gullberg et al., 1986). How EDN and ECP kill Purkinje cells is not clear but ribonuclease activity appears to be involved (Sorrentino et al., 1992).

Onconase, a protein currently in clinical trials for cancer therapy, was recently sequenced (Ardelt et al., 1991) and found to be a member of the RNase superfamily 30% identical to RNase A and approximately 33% identical to EDN and ECP. Onconase was originally isolated from extracts of *Rana pipiens* early embryos based on its antiproliferative/cytotoxic activity against cancer cell lines *in vitro* (Darzynkiewicz et al., 1988) and *in vivo* (Mikulski et al., 1990). The mechanism of onconase anticancer activity is unknown.

We demonstrate here that onconase causes the Gordon phenomenon when injected intraventricularly into guinea pigs. In contrast, bovine pancreatic RNase A has no effect on the guinea pigs at doses 5000 times greater than the LD_{50} of EDN or onconase. Although onconase is orders of magnitude less active enzymatically than EDN or RNase A at physiological pH, its ability to cause the Gordon phenomenon is equal to that of EDN. Chemical modification of onconase leading to 70% and 98% elimination of RNase activity leads to a commensurate decrease in its ability to cause the Gordon phenomenon, indicating that ribonuclease activity of onconase is required for Purkinje cell toxicity and the Gordon phenomenon. In addition, ribonuclease activity also appears to be involved in the cytotoxic activity of onconase against tumor cell lines *in vitro*, as shown by the correlation between the loss of the ability of onconase to

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inhibit protein synthesis of cells and the loss of ribonuclease activity (Ardelt et al., 1991; Wu et al., 1993). Thus, a new mechanism for neurotoxicity and cellular toxicity is suggested: the degradation of cellular RNA.

Materials and Methods

Onconase and EDN were purified as described (Ackerman et al., 1983b; Ardelt et al., 1991). Bovine pancreatic RNase A and highly polymerized RNA were purchased from Calbiochem or Worthington.

Chemical modification of onconase. Alkylation of onconase with iodoacetate was performed according to a modified method of Crestfield et al. (1963). Onconase (1 mM) was incubated with a 50- or 9.6-fold molar excess of sodium iodoacetate (Aldrich) in 0.1 M sodium acetate buffer (pH 5.5) for 18 hr at 23°C. The samples were desalted on a Bio-Gel P-2 (Bio-Rad) column in 5% (v/v) formic acid and lyophilized.

Intrathecal injections of RNases. RNases were injected intraventricularly into female Hartley guinea pigs (350–400 gm) obtained from Charles River Breeding Laboratories and anesthetized with intraperitoneal injections of ketamine and xylazine (80–85 mg/kg and 10 mg/kg, respectively). A midline sagittal incision from the inion to the laminal arch of C1 was made in guinea pigs in a stereotaxic frame. The fascia and underlying muscle were dissected away, exposing the membrane and underlying cisterna magna. With the use of an operating microscope, the dura overlying the cisterna magna was carefully pierced with a 30 gauge needle and the RNase (20–100 μl in PBS) was slowly injected into the subarachnoid space. The overlying dissected muscle was sutured and the skin incision closed with autoclips. The incision was treated with Furazolidone aerosol powder (Veterinary Products Laboratories). Animals were observed daily for 2 weeks, then periodically for 1–2 months, and were treated according to the National Institutes of Health guidelines. Several animals were anesthetized and perfused with 10% neutral buffered formalin fixative. The whole brains were removed and embedded in paraffin. Sagittal step sections were taken and stained with hematoxylin/eosin.

Neurological symptomology. The Gordon phenomenon was determined as described (King, 1939) by placing the animals on their backs and observing their ability to obtain an upright position. As the Gordon phenomenon advances, it takes the animal longer and longer to resume the upright position. In the more severe cases, one of the hind limbs may become paralyzed and the animal must be killed within 1–3 d after the onset of this symptom. Some animals also demonstrated a slight tremor when they tried to walk. Animals with mild neurological deficit recovered after several days of the early symptoms, whereas those with a moderate Gordon phenomenon exhibited symptoms for several weeks.

RNase assay. RNase activity was determined at pH 6.0 and 7.5. The assay at pH 7.5 contained, in a final volume of 0.3 ml, 0.33 mg/ml highly polymerized yeast RNA, the appropriate concentration of ribonuclease (dilutions made in 0.5 mg/ml human serum albumin, Calbiochem), and 0.2 M Tris-HCl, pH 7.5, 0.2 mM EDTA, 0.2 mg/ml human serum albumin. The incubation mixtures (0.3 ml) at pH 6.0 contained 0.56 mg/ml highly polymerized yeast RNA, 20 μg/ml human serum albumin, 0.2 M MES (2[*N*-morpholino]ethanesulfonic acid) buffer, pH 6.0, and the appropriate concentrations of ribonuclease diluted as above. The mixtures were incubated for 15 min at 37°C and the reaction was terminated with 700 μl of 3.4% ice-cold perchloric acid. The remaining steps were as described (Rybak et al., 1991).

Protein synthesis assay. The effect of the various RNases on protein synthesis of K562 cells, a human erythroleukemia cell line, was measured as described (Newton et al., 1992).

Results and Discussion

When EDN or ECP is injected intraventricularly into rabbits or guinea pigs, they cause the Gordon phenomenon, characterized by muscular rigidity, ataxia, paralysis, and tremor that can result in death (Durack et al., 1981; Fredens et al., 1982). EDN injected into the cerebral spinal fluid via the cisterna magna resulted in the Gordon phenomenon with all the characteristic neurological symptoms. As shown in Table 1, EDN caused the Gordon phenomenon in guinea pigs with an IC_{50} of 2 μg. Injection of 10 μg resulted in severe neurotoxicity in all guinea pigs, which led to death in 4 d. Similar neurotoxicity leading to death was observed by Fredens et al. (1982). The LD_{50} for EDN was 4 μg per animal (Fig. 1). We examined the neurotoxicity of RNase A and onconase, two homologs of EDN and ECP. As shown in Table 1 and Figure 1, the intraventricular injection of as much as 20 mg of bovine pancreatic RNase A did not result in neurotoxicity as measured both by the neurologic symptoms of weakness and slight incoordination, shown best by the reduced ability to gain the upright position when the animal is placed on its back, as well as by the survival of animals. The animals demonstrated no ataxia, tremor, or other neurologic symptoms and appeared healthy. This is a dose 5000 times greater than that of EDN needed to give an LD_{50} (4 μg/animal) and 10,000 times greater than the amount of EDN (2.0 μg/animal) where 50% of the animals developed neurologic symptoms of the Gordon phenomenon. Sorrentino et al. (1992) also report that RNase A does not cause the Gordon phenomenon in rabbits at a dose of 200 μg per rabbit.

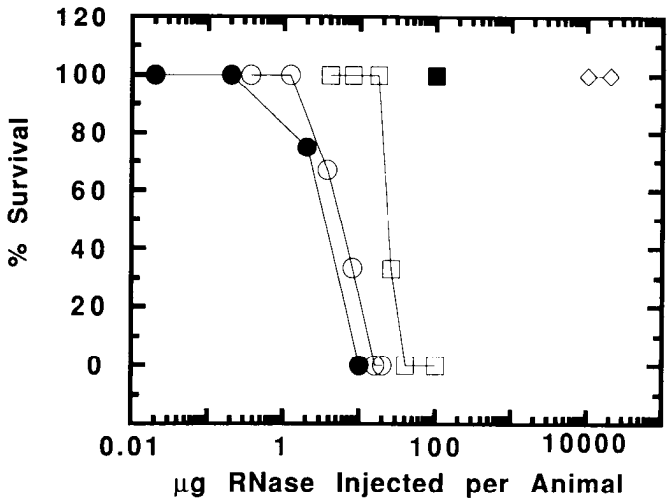

Figure 1. Intrathecal injection of RNases into guinea pigs. Guinea pigs were injected into the CSF with the indicated doses of EDN (*solid circles*), onconase (*open circles*), bovine pancreatic RNase A (*open diamonds*), modified onconase retaining 30% RNase activity (*open squares*), and modified onconase retaining 2% RNase activity (*solid square*) as described in Materials and Methods. Neurological symptoms and survival of animals were monitored. Plotted is the percentage survival of animals. Data points represent three to six animals per group except for RNase A, in which there were two animals per group.

Unlike RNase A but similar to EDN, the intrathecal administration of onconase resulted in animal toxicity (Table 1, Fig. 1). Animals injected with as little as 0.36 μg of onconase displayed ataxia and tremor (Table 1). The animals had weakness in the hind legs and difficulty righting. At the lower doses of onconase (0.36–3.7 μg), many of the animals exhibiting the Gordon phenomenon eventually regained normal neurological function, as has also been reported for the low doses of eosinophil lysates (Durack et al., 1979). With increasing doses animals developed more severe neurologic symptoms, such as inability to right themselves or walk and severe stiffness in the hind limbs. Animals died or had to be killed with an LD_{50} of 5 μg per animal (Fig. 1). The neurologic symptoms of animals treated with onconase were identical to those of animals treated with EDN. Histological examination of the cerebellum of onconase-treated animals demonstrated extensive loss of Purkinje cells compared to those animals injected with saline (compare Fig. 2*a,c* with Fig. 2*b,d*). In severely affected areas, holes appeared where Pur-

Table 1. Neurotoxicity of RNases

RNase	Dose (μg)	Number with symptoms[a]	Number survived	Days to death	Number days observed
EDN	0.02	0/4	4/4		17
	0.2	0/4	4/4		17
	2.0	2/4	3/4	9 (1)[b]	17
	10.0	3/3	0/3	4 (3)	4
Onconase	0.36	2/3	3/3		12
	1.0	2/6	6/6		14
	3.7	8/9	6/9	7 (1), 16 (2)	16
	8.0	2/3	1/3	5 (2)	23
	16.0	3/3	0/3	3 (1), 4 (2)	4
	20.0	3/3	0/3	3 (2), 4 (1)	4
	36.0	3/3	0/3	2 (2), 7 (1)	7
Alkylated onconase[c] (30% active)	4.0	3/4	4/4		11
	8.0	0/3	3/3		15
	17.0	0/3	3/3		24
	26.0	2/3	1/3	5 (2)	23
	40.0	3/3	0/3	4 (1), 5 (2)	5
	100.0	3/3	0/3	3 (3)	3
Alkylated onconase[c] (2% active)	99.9	0/3	3/3		18
RNase A	10,000.0	0/3	3/3		18
	20,000.0	0/2	2/2		18

[a] RNases were injected intrathecally into guinea pigs and their neurological symptoms measured as described in Materials and Methods.

[b] Numbers in parentheses indicate the number of animals that died on that day.

[c] Onconase was chemically modified as described in Materials and Methods.

kinje cells were previously located. EDN also causes a selective loss of Purkinje cells (Fredens et al., 1982). Furthermore, like the results reported by Durack et al. (1979, 1981) for rabbits treated with EDN, there was a spongy vacuolation of the white matter of the cerebellum in guinea pigs treated with onconase. Therefore, based upon neurologic symptoms and the loss of Purkinje cells, onconase appears to cause the Gordon phenomenon.

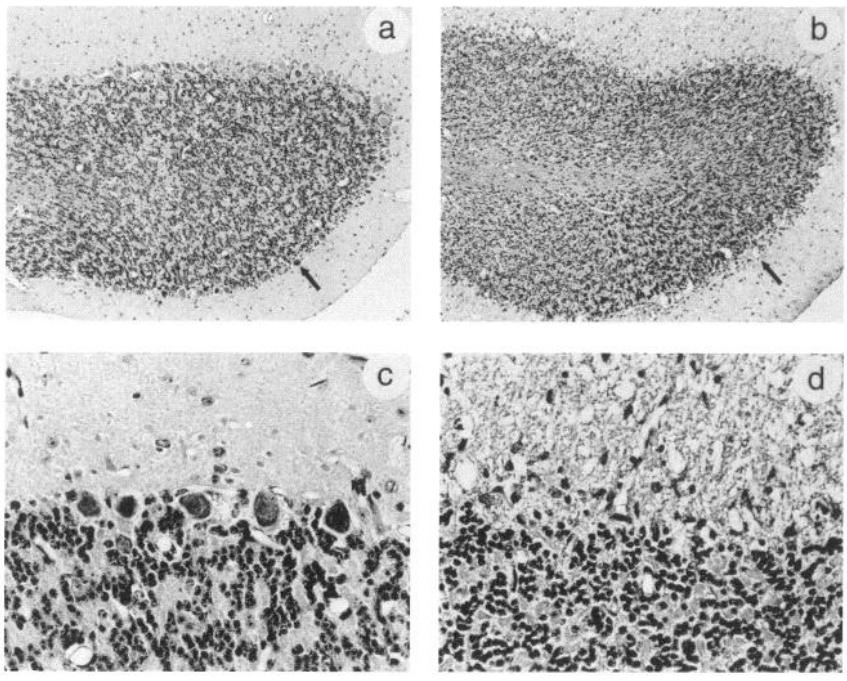

Figure 2. Photomicrography of cerebellar folia from a control animal (*a* and *c*) or an onconase-treated animal (*b* and *d*): low (100×) (*a, b*) and high (400×) (*c, d*) magnification of animals injected intraventricularly with either PBS or onconase (3.6 μg) as described in Materials and Methods. Purkinje cells (*a*) or holes previously occupied by Purkinje cells (*b*) are indicated by the *arrows*.

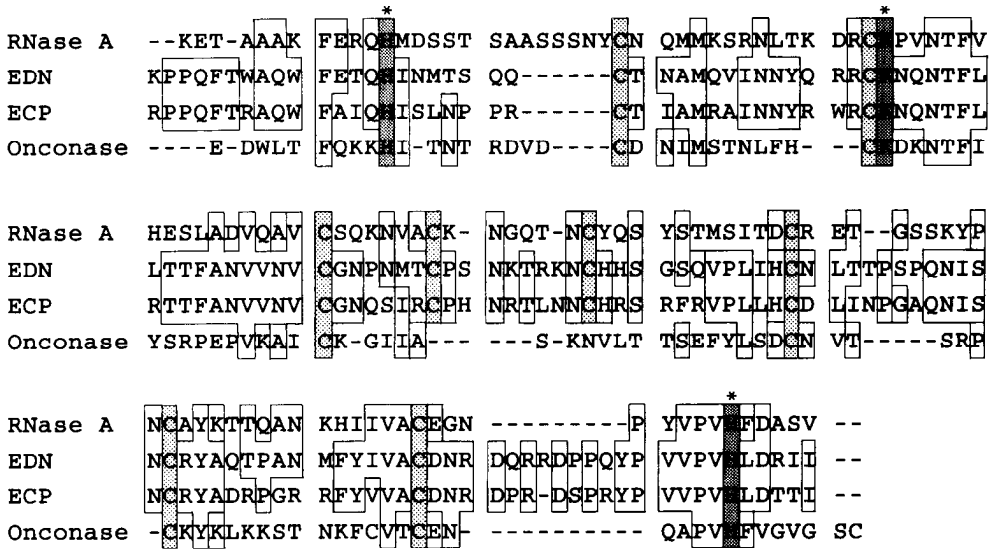

Figure 3. Sequence alignment of the amino acid sequences of EDN (Rosenberg et al., 1989b), ECP (Rosenberg et al., 1989a), and onconase (Ardelt et al., 1991; Mosimann et al., 1992) with those of bovine pancreatic RNase A (Beintema et al., 1988). Regions of identical sequence are enclosed in *boxes*; cysteine residues are in *lightly shaded boxes* and the putative catalytic histidine and lysine residues are in *heavily shaded boxes and marked by an asterisk. Dashes* represent gaps introduced to align cysteine residues and the catalytic residues. Residues are numbered according to bovine pancreatic RNase A.

Comparing the sequences of onconase, RNase A, EDN, and ECP reveals that there is a close homology in primary structure among the four proteins (Fig. 3). Three of the four disulfide bonds of RNase A are conserved in onconase and all four disulfide bonds of RNase A are conserved in EDN and ECP. Onconase has an extra disulfide loop at the C-terminus not found in the other three proteins. The three key active site residues involved in enzymatic activity of RNase A, H12, K41, and H119, are all conserved in the four proteins. Close examination of the amino acid sequence of the four proteins does not reveal any region common to EDN, ECP, and onconase that is lacking in RNase A that might explain the different abilities to cause the neurotoxicity. The extra nine amino acid region found in EDN and ECP that is lacking in RNase A is also lacking in onconase (between residues 113 and 114, Fig. 3) and thus cannot account for the neurotoxicity. The basicity of the ribonucleases has been suggested to predict neurotoxic activity (Sorrentino et al., 1992). As shown in Table 3 and in Sorrentino et al. (1992), bovine pancreatic RNase A has the lowest net positive charge (four excess basic amino acids) and lacks the ability to cause the Gordon phenomenon. EDN is both more positively charged (seven excess basic amino acids) and more neurotoxic than RNase A. Onconase, on the other hand, is as neurotoxic as EDN but has only five basic amino acids in excess, intermediate between RNase A (lacking neurotoxicity) and human pancreatic RNase (approximately 14–20% as neurotoxic as EDN), which contains six excess basic amino acids (Sorrentino et al., 1992). Therefore, the results with onconase show that basicity does not correlate with RNase cytotoxicity to Purkinje cells. The determination of the number of excess basic amino acid residues does not define the charge distribution on the surface of the molecules, which must be quite varied since EDN has the lowest isoelectric point (pI 8.9) (Rosenberg et al., 1989a) compared to RNase A (pI 9.4) (Richards and Wyckoff, 1971) and onconase (pI >9.5) (W. Ardelt, unpublished observation). Our results also show that glycosylation, an important structural difference between EDN, ECP, and RNase A, does not mediate neurotoxicity because onconase completely lacks carbohydrate (see Table 3) (Beintema et al., 1988; Ardelt, unpublished observation), whereas EDN and ECP are glycosylated to varying degrees depending on the tissue of origin (Beintema et al., 1988).

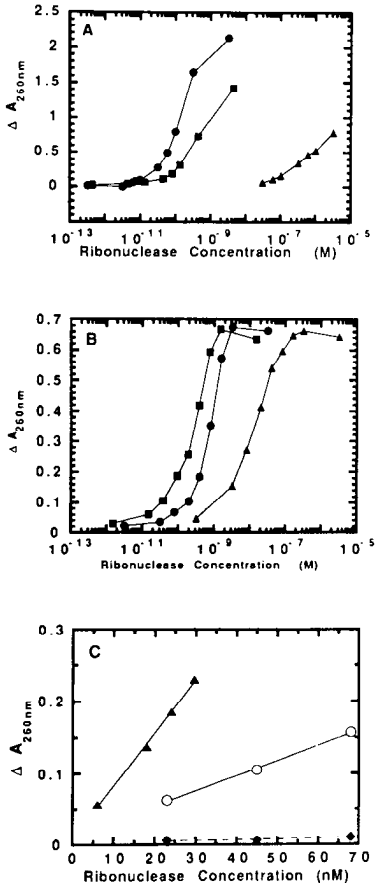

Figure 4. RNase activity of bovine pancreatic RNase A, EDN, onconase, and the two alkylated onconases. RNase activities determined at pH 7.5 (*A*) or pH 6.0 (*B* and *C*) were measured as described in Materials and Methods using highly polymerized yeast RNA as the substrate. *Solid circles*, bovine pancreatic RNase A; *solid squares*, EDN; *solid triangles*, onconase; *open circles*, alkylated onconase retaining 30% ribonuclease activity; *solid diamonds*, alkylated onconase retaining 2% ribonuclease activity.

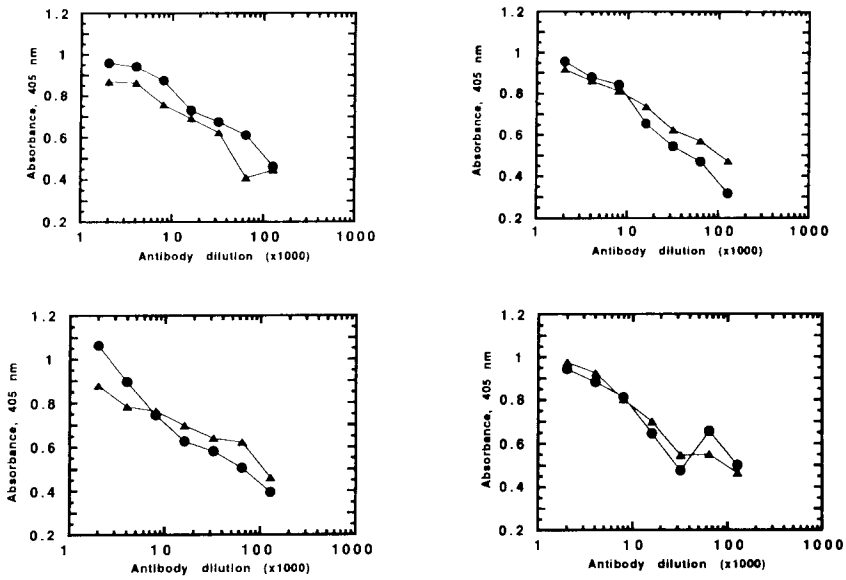

Figure 5. Radioimmunological assays of onconase and alkylated onconase. Antibodies against onconase were raised in four rabbits and tested in an ELISA assay for their abilities to recognize native onconase and onconase that had been modified with iodoacetate leading to a 96% elimination of ribonuclease activity. ELISA assays were performed as described (Tijssem, 1985). Each panel represents an independent animal antiserum. No significant difference between onconases is noted for binding of the antibodies to multiple epitopes on the ribonucleases. *Circles*, native onconase; *triangles*, alkylated onconase.

The enzymatic activity of RNase A has been extensively probed by chemical modification (reviewed in Richards and Wyckoff, 1971). Iodoacetate treatment modified H12 and/or H119 in the active site of RNase A and inactivated enzyme activity (Crestfield et al., 1963; Richards and Wyckoff, 1971). Treatment of onconase with iodoacetate also resulted in the modification of one of the three histidyl residues (Ardelt, unpublished observations). The extent of modification depended upon the molar ratio of iodoacetate to onconase. Enzyme activity of two batches of onconase, modified to different extents, was found to be 30% and 2% of the native enzyme (Fig. 4*C*). Individual ELISA assays using polyclonal antibodies raised in four rabbits demonstrated that there was no significant difference in the reactivity of the antibodies between the native and alkylated onconases (Fig. 5), indicating that protein conformation was unaffected by iodoacetate treatment.

We examined the neurotoxicity of these two preparations in guinea pigs. As shown in Table 1, 26 μg of the modified onconase that retained 30% enzymatic activity was approximately 30% as neurotoxic as native onconase (8 μg). The LD_{50} for the onconase retaining 30% activity was 25 μg, compared to 5 μg noted for onconase (Fig. 1). The alkylated onconase containing 2% ribonuclease activity was not active when administered at a dose as high as 98 μg per animal, thus at least 22-fold less active than native onconase. Therefore, there is a very close correlation between the extent of ribonuclease activity of onconase and the extent of neurotoxicity, indicating that ribonuclease activity is required to cause the Gordon phenomenon. Similar results were recently reported for EDN (Sorrentino et al., 1992).

Table 2. Enzymatic activities of the ribonucleases[a]

Ribonuclease	pH	ΔA_{260nm}/pmol
RNase A	7.5	24.9 ± 1.8
EDN	7.5	7.9 ± 1.5
Onconase	7.5	0.004[b]
RNase A	6.0	1.23 ± 0.25
EDN	6.0	1.94 ± 0.38
Onconase	6.0	0.04 ± 0.02

[a] The change in absorbance at 260 nm was determined for each given enzyme under the conditions described in Materials and Methods. The numbers were calculated from the linear parts of the enzyme curves and represent the average of triplicate determinations ± SD except where noted. The concentrations of each respective enzyme were determined spectrophotometrically using the following extinction coefficients: RNase A, $E^{1\%}_{280nm}$ = 7.3; EDN, $E^{1\%}_{280nm}$ = 15.5; onconase, $E^{1\%}_{280nm}$ = 8.8.

[b] This number represents the average of duplicate determinations.

Ribonuclease activity of EDN, RNase A, and onconase was measured in order to examine the relationship between Purkinje cell toxicity and the specific activities of the RNases. Ribonuclease assays were performed at two different pHs: the physiologic pH of 7.5 that is close to the optimal pH determined for RNase A (Richards and Wyckoff, 1971; Lee and Vallee, 1989) and pH 6.0, which is the optimal pH for onconase (Ardelt, unpublished observation). As shown in Figure 4 and Table 2, the ribonuclease activity of RNase A and EDN is greater than that of onconase whether determined at pH 7.5 or pH 6.0. The difference in activities between RNase A and onconase is greater at physiological pH. Other RNA substrates, tRNA or yeast RNA, showed similar results at pH 7.5 (data not shown) to those obtained with the highly polymerized yeast RNA shown in Figure 4*A*. Similar results have been reported by Ardelt et al. (1991). Another ribonuclease, ECP, is more potent than EDN in causing the Gordon phenomenon in guinea pigs (Fredens et al., 1982) and is less active enzymatically (Gullberg et al., 1986). Although ribonuclease activity appears to be necessary for causing the Gordon phenomenon, other properties of the RNases also play roles in the neurotoxicity.

Onconase was originally isolated from frog embryo extracts because it exhibited antitumor properties (Darzynkiewicz et al., 1988; Mikulski et al., 1990; Ardelt et al., 1991). The cytotoxicity of onconase to various cells *in vitro* was determined because it directly examines cellular cytotoxicity. Toxicity was determined by comparing the abilities of the various ribonucleases to inhibit protein synthesis of K562 cells, a human erythroleukemia cell line. As shown in Figure 6, onconase inhibits protein synthesis with an IC_{50} of 0.4 μM, which is similar to the LD_{50} determined for neurotoxicity [0.4–0.6 μM, assuming a guinea pig cerebrospinal fluid (CSF) volume of 0.5 ml]. As noted for the Gordon phenomenon, chemically modified onconase with decreased ri-

bonuclease activity was correspondingly less toxic to K562 cells (Fig. 6), confirming *in vitro* that onconase cytotoxicity is dependent upon ribonuclease activity (Ardelt et al., 1991). RNase A only inhibited protein synthesis in K562 cells at concentrations greater than 100 μM. EDN, on the other hand, is identical to onconase in neurotoxicity (LD_{50} 0.5 μM) yet is 35 times less active than onconase in protein synthesis inhibition of K562 cells (IC_{50} 14 μM). The low *in vitro* cytotoxicity for EDN is also noted for other cell types. EDN at 14 μM or 24 μM had no effect *in vitro* on tracheal epithelium (Motojima et al., 1989) or Hep G2 cells (data not shown), respectively. Molina and Kierszenbaum (1988) report that EDN at 50 μM is cytotoxic to rat heart myoblasts.

RNase A can be toxic to cells when placed directly inside the cell by microinjection (Rybak et al., 1991) or when conjugated to proteins capable of entering cells, such as transferrin, or antibodies to the transferrin receptor or to the T cell antigen CD 5 (Rybak et al., 1991; Newton et al., 1992), showing that if RNase A can enter cells, it is toxic. EDN and onconase may contain some molecular domains or three-dimensional structure characteristics that could determine their entry into Purkinje cells that are lacking in RNase A. Another explanation for the greater neurotoxicity of EDN and onconase than RNase A may be the rate of intracellular metabolism. Studies by Dice (1990) demonstrate that RNase A contains a peptide motif that directs RNase A to the lysosomes in response to serum deprivation. This sequence is KFERQ at amino acid residues 7–11 in RNase A (Fig. 3). This sequence is not found in EDN, ECP, or onconase. Another explanation for the vastly different cytotoxicities may be the sensitivity of the ribonucleases to ribonuclease inhibitors (Table 3). RNase A is inhibited by a ribonuclease inhibitor (Blackburn et al., 1977), and thus may become inactivated within the cell, whereas onconase is not affected by the inhibitor and therefore would be expected to retain full enzymatic activity within a mammalian cell (Wu et al., 1993). Although EDN is also sensitive to the ribonuclease inhibitor, Sorrentino et al. (1992) demonstrate that more inhibitor is required to inhibit EDN enzymatic activity than to inhibit RNase A activity. Finally, the different cytotoxicities may be due to the particular substrate that is hydrolyzed within a cell by the different ribonucleases (Saxena et al., 1992; S. R. Rybak, unpublished observation).

Thus, it appears that EDN is more toxic to Purkinje cells *in vivo* relative to other cell types *in vitro*. Why Purkinje cells are so sensitive to certain RNases is unknown. Purkinje cells have been shown to extract selectively some molecules from the CSF (Ivy et al., 1984; Mares et al., 1984; Borges et al., 1985; Karpiak and Mahadik, 1987). The physiological significance of this selective uptake is unknown but may contribute to the loss of Purkinje cells in certain disease states. Ribonucleases are not the only proteins that cause Purkinje cell loss. Riedel et al. (1990) demonstrated that a diphtheria toxin mutant (CRM 107) lacking the receptor-binding activity and CRM 107 covalently coupled to monoclonal antibodies cause the characteristic neurological symptoms of the Gordon phenomenon as well as the selective killing of Purkinje cells after intrathecal injection. Similar results have also been reported for an anti-Thy 1.1-saporin conjugate (Davis and Wiley, 1989).

The significance of the Gordon phenomenon in man is not clear. Various neurological abnormalities have been reported in patients exhibiting high levels of eosinophils, EDN, and ECP (reviewed in Ackerman et al., 1983a; Venge et al., 1983; Youle

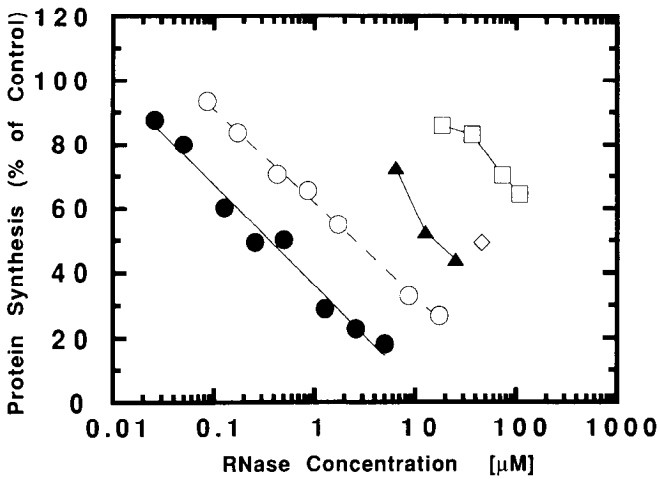

Figure 6. Toxicity of the RNases to K562 cells, a human erythroleukemia cell line. Cells were incubated either overnight [bovine pancreatic RNase A (*open squares*), onconase (*solid circles*), alkylated onconase retaining 30% ribonuclease activity (*open circles*), alkylated onconase retaining 2% ribonuclease activity (*open diamond*)] or for 48 hr [EDN (*solid triangles*)]. Protein synthesis was determined as described in Materials and Methods.

Table 3. A summary of the properties of the ribonucleases

Ribonuclease	Neurotoxicity *in vivo*[a]	Enzymatic activity[b]	Glycosylation[c]	Ribonuclease inhibitor sensitivity[d]	Toxicity *in vitro*[e]	Excess basic amino acids[f]
RNase A	–	+++	–	++	–	4
Onconase	++	+	–	–	++	5
EDN	++	+++(+)	+	+	+	7

[a] Neurotoxicity is defined as the induction of the Gordon phenomenon noted in Table 1.

[b] Enzymatic activity is that shown in Figure 4.

[c] From Beintema et al. (1988) and Ardelt (unpublished observations).

[d] From Blackburn and Moore (1982), Sorrentino et al. (1992), Wu et al. (1993), and Ardelt (unpublished observations).

[e] *In vitro* toxicity is shown in Figure 5 and from Darzynkiewicz et al. (1988), Molina and Kierszenbaum (1988), Motojima et al. (1989), Rybak et al. (1991), and Wu et al. (1993).

[f] Excess basic amino acids were determined by subtracting the total number of aspartic and glutamic acids from the total number of arginines and lysines. Amino acid sequences are from the following references: RNase A, Beintema et al. (1988), onconase, Ardelt et al. (1991), EDN, Rosenberg et al. (1989b).

et al., 1993). Onconase therapy for cancer has caused no neurological abnormalities that can be attributed to a Purkinje cell loss for those patients treated systemically by intravenous administration of doses of onconase up to 960 $\mu g/m^2$ (Mikulski et al., 1993). Phase 1 clinical trials of onconase for cancer treatment in a broad spectrum of human solid tumors have been completed and currently Phase 2 trials are being conducted.

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