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Toxicity and cytotoxicity of nigrin b, a two-chain ribosome-inactivating protein from *Sambucus nigra*: comparison with ricin

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Abstract Nigrin b, a lectin isolated from the bark of elderberry (*Sambucus nigra* L.), has structure and enzymatic activity similar to that of ricin and other type 2 ribosome-inactivating proteins (RIPs), and yet is much less toxic to cells and animals. In an attempt to explain this difference, we studied (1) the cytotoxicity of both lectins at 18 and 37 °C, and in the presence of substances interfering with intracellular routing, and (2) the binding of nigrin b to, and its uptake and degradation by HeLa cells, in parallel with ricin. As compared with the latter, (1) less nigrin b was bound and more was degraded by cells, with a resulting lower concentration remaining inside the cells, and (2) there is evidence for a different intracellular routing followed by the two lectins. These results may explain at least partly the different cytotoxicity and consequently the lower toxicity to mice of nigrin b compared with ricin.

Key words Nigrin b · Ribosome-inactivating proteins · Lectins · HeLa cells · Toxicity to mice

Introduction

Ribosome-inactivating proteins (RIPs) are divided into two groups: type 1, consisting of a single polypeptide chain which enzymatically damage ribosomes, and type 2, in which an enzymatically active A chain is linked by a disulphide bridge to a larger B chain with the properties of a galactose-specific lectin (reviewed by Barbieri et al. 1993). Until recently the known type 2 RIPs included ricin and similar toxins, with much higher toxicity than type 1 RIPs. The different potency was ascribed to the presence of the B chain, which is able to bind to cells and, at least in the case of ricin, also facilitates the entry of the molecule inside the cells. However in the last few years, some galactose-specific lectins from different tissues of *Sambucus nigra* L. (elderberry; Girbés et al. 1993a; 1996; Citores et al. 1994; Van Damme et al. 1996), *S. ebulus* L. (Girbés et al. 1993b) and *Eranthis hyemalis* (winter aconite; Kumar et al. 1993) were found to be classified as type 2 RIPs, and are still much less toxic to cells than ricin.

Nigrin b is a galactose-specific lectin isolated from the bark of elderberry, with two-chain structure and enzymatic activity similar to ricin (Girbés et al. 1993a). Both ricin and nigrin b depurinate mammalian ribosomes, a characteristic property of RIPs (reviewed by Endo 1988), thus inhibiting cell-free protein synthesis. The two lectins, however, differ in their effect on whole cells: ricin inhibits protein synthesis by HeLa cells with an IC_{50} (concentration causing 50% inhibition) in the ng/ml concentration range (reviewed by Barbieri et al. 1993), whereas the IC_{50} of nigrin b is in the order of μg/ml (Girbés et al. 1993a). In agreement with this finding, the lectins differ also in toxicity to animals, nigrin b not being toxic to mice at the dose of 1.6 mg/kg of body wt. (Girbés et al. 1993a), whereas ricin has an LD_{50} of 8 μg/kg (Olsnes and Pihl 1973). The reason(s) for the lower toxicity of some type 2 RIPs could reside (1) in an insufficient binding to, and/or uptake by cells, (2) in a different intracellular routing, or (3) in a more rapid degradation once inside the cells or (4) in a lower affinity for the substrate GAGA sequence.

The present investigations were undertaken with the aim of ascertaining the possible reason(s) for the different toxicity of lectins having similar structure and properties, such as nigrin b and ricin. The cytotoxicity of

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The experiments reported here were conducted according to "The Guide for the Use of Laboratory Animals" as prepared by the Committee on the Care and Use of Laboratory Animals, National Research Council, NHI Publication no. 80–23 (1985) and following the national guidelines for the care and use of laboratory animals.

both lectins was studied at 18 and 37 °C, and in the presence of substances interfering with intracellular routing. The binding of these lectins to, their entry into, and degradation by, HeLa cells were studied and compared. The LD_{50} to mice of nigrin b was determined.

Materials and methods

Inhibition of protein synthesis

Nigrin b was prepared as described by Girbés et al. (1993a) and ricin as described by Nicolson et al. (1974). The effect of lectins on cell-free protein synthesis was determined with a rabbit reticulocyte lysate as described by Parente et al. (1993). Reaction mixtures contained, in a final volume of 62.5 µl: 10 mM TRIS/HCl buffer, pH 7.4, 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 3 µg of creatine kinase, 0.05 mM amino acids (minus leucine), 89 nCi of L-[^{14}C]leucine (240 Ci/mol), and 25 µl of rabbit reticulocyte lysate. Incubation was at 28 °C for 5 min. Lectins were reduced by incubation at 37 °C for 1 h with 2% 2-mercaptoethanol immediately prior to assay.

The effect on cellular protein synthesis was studied with HeLa cells maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine and antibiotics (complete medium) at 37 °C in a 5% CO_2 atmosphere. HeLa cells were seeded into 24-well plates ($0.5–1 \times 10^5$ cells/ml, 0.5 ml per well). After 24 h of incubation to allow monolayer formation, cells were washed twice each with 1 ml Hank's solution, and 0.5 ml of serum-free medium containing the appropriate concentration of RIP was added. After 18 h of incubation at the indicated temperature, cells were washed as described above, and the medium was replaced by 0.5 ml of serum- and methionine-free medium containing 1 µCi/ml of [^{35}S]methionine (1164 Ci/mmol). After 2 h, or as otherwise specified, at 37 °C protein synthesis was terminated by adding 1 ml of 5% trichloroacetic acid (TCA), and the radioactivity incorporated was determined as described by Sandvig and Olsnes (1982). To assess the effects of galactose, chloroquine, NH_4Cl and brefeldin A, cells were preincubated with these substances for 1 h and then ricin or nigrin b in 10 µl was added and after 18 h of further incubation protein synthesis was determined as described. The concentration of lectins causing 50% inhibition of protein synthesis (IC_{50}) was calculated by linear regression analysis.

Binding to, uptake and degradation by cells

Nigrin b and ricin were labelled with ^{125}I by the Iodogen reagent as described by Fraker and Speck (1978). The day before the experiments, cells were harvested after 2 min at 37 °C with 0.5% trypsin in the presence of 0.2% EDTA and seeded in 24-well trays. Binding to, uptake and degradation by HeLa cells was determined as described by Battelli et al. (1996) with minor modifications. Briefly, the cells (0.5×10^6) were incubated at 37 °C for the indicated time in 0.5 ml HEPES-buffered complete medium with 10^{-8} M ^{125}I-labelled toxins ($1.18 \pm 0.18 \times 10^5$ cpm/5 pmol nigrin b and $0.49 \pm 0.05 \times 10^5$ cpm/5 pmol ricin). The uptake was stopped by placing the culture on ice. The medium was removed, adjusted to 10% TCA (w/v, final concentration) and centrifuged to determine the acid-soluble radioactivity as a measure of the degradation and exocytosis of degraded toxins.

The cells were washed once with cold complete medium and three times with phosphate-buffered saline, pH 7.5, and were stripped for 30 min at 0 °C with 0.1 M glycine/HCl buffer, pH 2.0, containing 0.02 M NaCl, to evaluate the membrane-bound RIPs. The cells were then washed once with phosphate-buffered saline, pH 7.5, and extracted at 37 °C for 10 min with 0.1 M KOH to measure the intracellular accumulation of toxins. Parallel cultures were incubated at 0 °C and the values obtained were subtracted from those obtained at 37 °C, as a correction for background radioactivity. The concentration of nucleic acid was measured at A_{260} corrected for protein concentration by the formula: $62.9 \times A_{260} - 36 \times A_{280}$.

Toxicity to mice

The toxicity of nigrin b was determined by injecting i.p. five scaled doses of the lectin (5–27 mg/kg body wt.) dissolved in 0.15 M NaCl to groups of seven Swiss female mice (body wt. 30 g). The LD_{50} was calculated after 15 days by linear regression analysis.

Results

Inhibition of protein synthesis

Both nigrin b and ricin inhibited cell-free protein synthesis, with similar IC_{50} values of 2.61×10^{-10} M and 8.14×10^{-10} M, respectively. Protein synthesis by HeLa cells was inhibited with IC_{50} values differing by four orders of magnitude, being 10^{-12} M (0.06 ng/ml) for ricin and 5×10^{-8} M (3.2 µg/ml) for nigrin b (Table 1). The inhibition of cell protein synthesis by both ricin and nigrin b was greatly reduced by galactose, and was somewhat potentiated by chloroquine and NH_4Cl (Table 1). On the contrary, brefeldin markedly reduced the cytotoxicity of ricin, but had no significant effect on that of nigrin b (Table 1). Also, when the temperature of incubation with the RIPs was lowered to 18 °C the inhibitory effect of ricin was reduced (Fig. 1).

The values obtained at 18 and 37 °C were compared by ANOVA test. A significant two-factors interaction ($F = 11.61$; df 4/10; $P < 0.001$) was found between the temperature and the concentration of ricin. The effect of ricin was significantly different between the two temperatures by Bonferroni's test (Glantz 1987) (df 10; $0.001 < P < 0.01$) at the three highest concentrations ($4.20 < t < 8.25$). The inhibitory effect of nigrin b was not affected by the temperature (Fig. 1). A similar temperature-related difference was observed after 6 h of incubation with the lectins (results not shown).

Table 1 Effect of various substances on the inhibition of protein synthesis by HeLa cells caused by nigrin b or ricin. Parallel cultures grown in the absence of RIPs (controls) incorporated 233 645 cpm. In the absence of RIPs, cell protein synthesis was inhibited by 10% (galactose), 33% (ammonium chloride), 39% (chloroquine), and 88% (brefeldin A). The concentration of lectins inhibiting by 50% cell protein synthesis (IC_{50}) was calculated by linear regression analysis ($0.94 < R > 1.00$) from the triplicate values of two experiments (RIP Ribosome-inactivating protein)

Additions	Nigrin b	Ricin
	IC_{50} (ng/ml)	
None	3215	0.06
Galactose (50 mM)	38856	5.25
Ammonium chloride (20 mM)	1045	0.02
Chloroquine (25 µM)	518	0.01
Brefeldin A (5 µg/ml)	3442	19.33

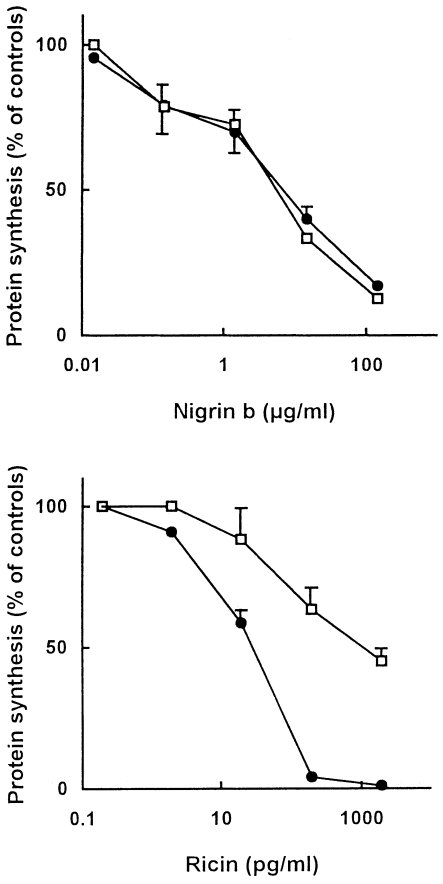

Fig. 1 Effect of temperature (18 °C, □; 37 °C, ●) on the inhibition of HeLa cells protein synthesis by nigrin b and ricin. Results are mean ± SE of triplicate values of 3 (nigrin b) and 2 (ricin) experiments

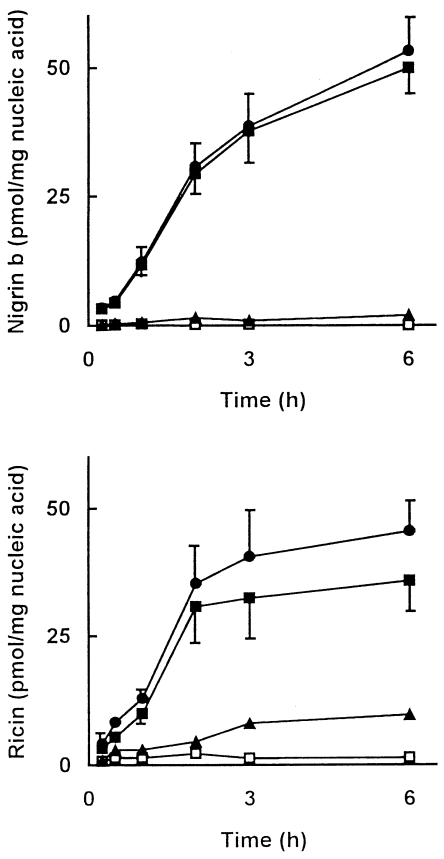

Fig. 2 Internalisation of nigrin b and ricin by HeLa cells. Cells were incubated for the indicated time, with 10^{-8} M ^{125}I-labelled toxins. Binding to (□), and intracellular accumulation (▲) and degradation by (■), HeLa cells of nigrin b and ricin were measured as described (Battelli et al. 1996). The internalisation (●) was calculated as the sum of the intracellular and degraded proteins. Results are given (mean ± SE) of 3 experiments in duplicate samples

Binding to, uptake and degradation by cells

HeLa cells bound 5.7 times more ricin than nigrin b after 1 h incubation, and after 6 h the internalised lectins were degraded by 94% (nigrin) and by 79% (ricin; Fig. 2). The ANOVA test was used to compare binding, internalisation, intracellular accumulation and degradation of nigrin b and ricin by HeLa cells. No significant difference between nigrin b and ricin was observed in the internalisation and degradation at different time intervals. However, the difference in the binding of the two RIPs to cell membrane ($F = 25.62$; df 1/24; $P < 0.0001$) and the interaction between intracellular accumulation of the two RIPs and time ($F = 7.56$; df 5/24; $P = 0.0002$) were significant. A progressively increasing significant difference by Bonferroni's test (df 24; $0.002 < P < 0.05$) was observed between the intracellular accumulation of nigrin and ricin ($2.32 < t < 7.43$) as early as after 0.5 h incubation. The difference in the degradation, although not statistically significant, contributes with the differences in binding to a significant difference in the amount of lectin remaining accumulated within the cells – 0.04% of the internalised nigrin versus 21% of the internalised ricin.

Toxicity to mice

Consistent with the reduced effect on cells compared to ricin, nigrin b was much less toxic than ricin to mice (Fig. 3). The LD_{50} value for nigrin B was 12 mg/kg, vs LD_{50} of 8 µg/kg of ricin (Olsnes and Pihl 1973). Deaths occurred between 2 (27 mg/kg) and 12 (10 mg/kg) days after injection.

Discussion

Consistently with previous results (Girbés et al. 1993a) the inhibitory effect of nigrin b on protein synthesis by HeLa cells was approx. 10^4 times lower than ricin, although both lectins had the same effect on cell-free protein synthesis. The present results show that nigrin b, which is required in much higher concentrations than ricin to inhibit protein synthesis in intact cells, binds to HeLa cells 5.7 times less than the latter, suggesting that these cells have a lower number of receptors for nigrin b than for ricin. It is known that the potency of ricin and other toxins is not proportional to the number of re-

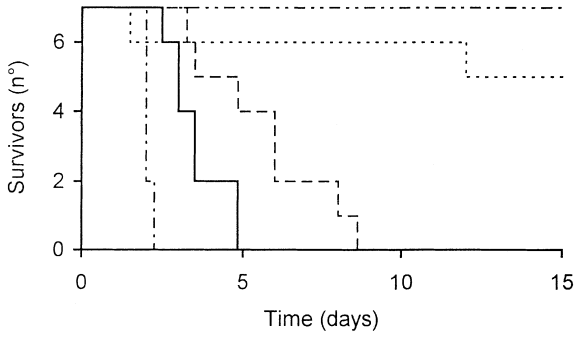

Fig. 3 Toxicity of nigrin b to mice. The lectin was administered i.p. dissolved in 0.15 M NaCl at the dose 5, 10, 16, 22 and 27 mg/kg body weight

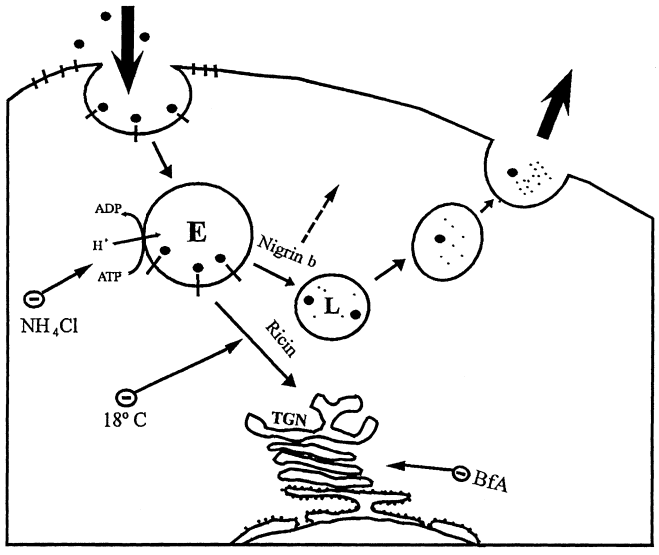

Fig. 4 Intracellular pathways followed by nigrin b and ricin. Symbols: E, endosome; L, lysosome; TGN, *trans*-Golgi network; BfA, brefeldin A; ●, proteins; (–) inhibitory effect; – – → pathway at high concentration; ——→ pathway at low concentration

ceptors present on the cell membrane. HeLa cells have more receptors for ricin ($1–3 \times 10^7$/cell; Sandvig et al. 1976) than for modeccin (2×10^5/cell; Olsnes et al. 1978), and yet modeccin is more potent than ricin. Thus the difference observed in the binding of nigrin b and ricin to HeLa cells is not sufficient to account for the difference in their toxicity. After internalisation 94% of nigrin b, compared with 79% of ricin, is degraded and extruded by the cells, suggesting that internalised nigrin b follows a different intracellular route than ricin. The combined effects of the differences in the binding and degradation may be the reason for the lower intracellular accumulation of nigrin b, compared to ricin, and consequently of its lower cytotoxicity.

To inhibit protein synthesis, ricin and related toxins must reach ribosomes in the cytosol. Endocytosed ricin was detected in endosomes, lysosomes, and *trans*-Golgi network (van Deurs et al. 1988). It was suggested that the bulk of ricin does not translocate through acidified endosomes, since agents which increase the pH of acidic vesicles increase the cytotoxicity of ricin presumably by reducing its degradation by lysosomes (Sandvig et al. 1979). The effect of weak amines on the toxicity to HeLa cells of nigrin b and ricin may be due, at least partly, to the interference with proton pump and the consequent rise in pH values. This, in turn, may be responsible for a lower degradation rate of both toxins in the lysosomal compartment because of the acidity requirement of hydrolases. Moreover, the pH may influence the conformation of proteins, and consequently their sensitivity to hydrolysis. The higher degradation rate of nigrin b by HeLa cells compared to ricin may depend on a higher affinity of nigrin b for proteolytic enzymes at low pH. Chloroquine may stimulate the cytotoxicity (1) by preventing the lysosomal degradation of nigrin b, thus leading to its faster accumulation above threshold levels for translocation into cell cytosol; (2) by changing the compartmentalization of ricin to the more favourable *trans*-Golgi network (reviewed by Ramakrishnan and Houston 1984).

The enhancement of the cytotoxicity of nigrin b by ammonium chloride and chloroquine suggests that this lectin may be processed via the lysosomal system, thus not reaching the ribosomes but being degraded and expelled from the cell. This concept is further supported by the different effect of temperature on the cytotoxicity of ricin and nigrin b. At 18 °C the cytotoxicity of nigrin b is not changed, whereas the effect of ricin is decreased compared with the effect at 37 °C. The latter observation was reported by van Deurs et al. (1985), who found also that at this temperature ricin does not reach Golgi elements and further supports the notion that after internalisation at 18 °C this lectin may take a route different from the Golgi network. The dependence of ricin cytotoxicity on the temperature suggests that for this substance to exert its effect a temperature-dependent step is required.

The temperature-dependent step is not present in the processing of nigrin b, consistently with a divergence in the pathways followed by the two lectins after reaching the lysosomal compartment. Moreover, ricin toxicity is greatly reduced by brefeldin A, suggesting that the toxin may migrate from endosomes through the Golgi network (Yoshida et al. 1991). This finding was confirmed in our experiments, and the fact that brefeldin A does not affect the cytotoxicity of nigrin b further suggests that this lectin does not utilise the Golgi apparatus but takes a different route. These conclusions are summarised in Fig. 4. Considered altogether, these observations (a) confirm that ricin must reach the Golgi apparatus to exert its full toxicity, and (b) suggest that, at least partly, nigrin b is less toxic to cells than ricin because of a different intracellular routing that does not include the *trans*-Golgi network. Finally, the different cytotoxicity may well account for the lower toxicity of nigrin b to mice, compared to ricin.

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