Ribosome-inactivating lectins with polynucleotide:adenosine glycosidase activity

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Abstract **Lectins from *Aegopodium podagraria* (APA), *Bryonia dioica* (BDA), *Galanthus nivalis* (GNA), *Iris* hybrid (IRA) and *Sambucus nigra* (SNAI), and a new lectin-related protein from *Sambucus nigra* (SNLRP) were studied to ascertain whether they had the properties of ribosome-inactivating proteins (RIP). IRA and SNLRP inhibited protein synthesis by a cell-free system and, at much higher concentrations, by cells and had polynucleotide:adenosine glycosidase activity, thus behaving like non-toxic type 2 (two chain) RIP. APA and SNAI had much less activity, and BDA and GNA did not inhibit protein synthesis.**

Key words: Lectin; Ribosome-inactivating protein; Polynucleotide:adenosine glycosidase; Protein synthesis

1. Introduction

Plant lectins are a heterogeneous group of proteins with highly specific recognition and binding properties. Although their biological function has not yet been unambiguously demonstrated, their preferential binding to glycoconjugates present on the surface of microbes or on epithelial cells along the intestinal tract of their potential predators has been interpreted as an argument in favour of a defence-related role (reviewed in [1]). The high affinity of plant lectins for animal glycans is not only important for their presumed defence role but is also the basis for their use in studying glycoconjugates and as cell-recognition molecules in biological and biomedical research.

Some lectins, in addition to their carbohydrate-binding domain(s), also possess a domain with a different biological activity. Thus, lectins belonging to the group of type 2 'ribosome-inactivating proteins' (RIP) consist of a sugar-binding B-chain linked to an active A-chain which irreversibly damages eukaryotic ribosomes through an enzymatic mechanism. The A-chains of type 2 RIP have similar enzymatic activity of, and are highly homologous to, the so-called type 1 RIP which consist of a single polypeptide of about 30 kDa and possibly evolved from a common ancestor (reviewed in [2]).

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Abbreviations: APA, *Aegopodium podagraria* agglutinin; BDA, *Bryonia dioica* agglutinin; GNA, *Galanthus nivalis* agglutinin; IRA, *Iris* hybrid agglutinin; RIP, ribosome-inactivating protein(s); SNAI, *Sambucus nigra* agglutinin 1; SNLRP, *Sambucus nigra* lectin-related protein

The enzymatic activity of type 1 RIP and of the A-chains of type 2 RIP was identified as an rRNA *N*-glycosidase (reviewed in [3]) which removes a single adenine from rRNA (A_{4324} from rat liver rRNA; A_{2660} from *Escherichia coli* rRNA). It was subsequently found that some RIP release more than one adenine from rRNA [4] and deadenylated RNA of other types, poly(A) and DNA [5–8], thus being polynucleotide:adenosine glycosidases.

Type 2 RIP were considered to be very potent toxins, the best known being ricin. The B-chains of these proteins bind to galactosyl-terminated receptors on the surface of most cells, allowing entry of the A-chain inside the cell where it inactivates ribosomes, with consequent arrest of protein synthesis and death of the cell. However, some lectins were identified which had structure, specificity and enzymatic activity similar to ricin and related toxins and still were scarcely toxic to cells. This group includes *Ricinus communis* agglutinin [9], nigrin b and SNAI from *Sambucus nigra* L. (elderberry) [10–13], ebulin 1 from *Sambucus ebulus* L. [14], the lectin from *Eranthis hyemalis* (winter aconite) [15] and cinnamomin from *Cinnamomum camphora* [16].

The presumed defensive role of some lectins may, at least in part, depend on their enzymatic activity. For instance, the antiviral activity of some type 2 RIP (reviewed in [17]) may depend not only on their ribosome-inactivating property but also on polynucleotide:adenosine glycosidase activity [5–8]. It is evident, however, that the carbohydrate-binding specificity of B-chain also strongly influences the toxicity of the type 2 RIP since it is the determining factor in recognition of the target cells.

The present investigation was undertaken to ascertain (i) whether some lectins with specificity for similar sugars (from *Aegopodium podagraria*, *Bryonia dioica*, *Iris* hybrid and *Sambucus nigra*) and a new lectin-related protein (from *Sambucus nigra*), all of which resemble type 2 RIP with respect to their heterodimeric structure, have RIP activity and, if so, (ii) whether they also exhibit polynucleotide:adenosine glycosidase activity. The study was extended to a mannose-specific lectin, *Galanthus nivalis* agglutinin, with homotetrameric structure. We observed that a lectin from *Iris* hybrid and a lectin-related protein from the bark of *Sambucus nigra* indeed have RIP and polynucleotide:adenosine glycosidase activities, with low cytotoxicity.

2. Materials and methods

2.1. Proteins

The following proteins were purified as described in the respective

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references: *N*-acetylgalactosamine-specific lectins from *Aegopodium podagraria* (APA) [18], *Bryonia dioica* (BDA) [19], and *Iris* hybrid (IRA) [20], the Neu5Ac(α2,6)Gal/GalNac-specific *Sambucus nigra* agglutinin I (SNAI) [13,21]; a *Sambucus nigra* lectin-related protein (SNLRP) [22]; the mannose-specific *Galanthus nivalis* agglutinin (GNA) [23]. Like type 2 RIP, IRA and BDA contain a single pair of unequal subunits. Whilst no sequence data are available for BDA, N-terminal sequencing of IRA demonstrated that its A-chain definitely has strong sequence similarity with the A-chain of ricin and abrin [20]: in particular, the residues G^{21}, F^{24} and R^{29} common to all RIP (numbers refer to ricin sequence) are present and aligned. It is not known, however, whether the active site residues characteristic of RIP have been conserved in IRA. SNAI has been identified as a type 2 RIP composed of four [A–s–s–B]-pairs [13]. SNLRP is built up of a single [A–s–s–B]-pair and inhibits cell-free protein synthesis but is devoid of carbohydrate-binding activity [22]. Molecular cloning demonstrated that both SNAI and SNLRP contain the active site residues characteristic of RIP [13,22]. APA, a 480 kDa octamer composed of two types of non-covalently-bound subunits with similar M_r, apparently differs from type 2 RIP but has amino acid composition similar to SNAI [21] and BDA [18]. No sequence data are available for APA. Finally, GNA which is a homotetramer of four 12 kDa subunits and has no sequence similarity with any RIP [23], was examined as an example of a lectin completely different from type 2 RIP.

2.2. Cell lines

The cell lines used, namely mouse 3T3 (fibroblasts), and human HeLa (carcinoma) and BeWo (chorioncarcinoma) were maintained as monolayer cultures in RPMI-1640 medium supplemented with antibiotics and 10% foetal calf serum (20% for BeWo cells), in a humidified atmosphere containing 5% CO_2 at 37°C. Subcultures were obtained by trypsin treatment of confluent cultures. The JM cell line (monocyte-derived) was grown in suspension in the same medium and treated with phorbolmyristate acetate to induce adhesion as described [24].

2.3. Protein synthesis

Protein synthesis was determined with a cell-free system (a rabbit reticulocyte lysate) as described previously [24]. 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. When indicated, lectins and SNLRP were reduced by incubation at 37°C for 30 min with 20 mM 2-mercaptoethanol immediately prior to assay.

Protein synthesis by various cell lines was assayed as described previously [25]. Cells (10^5/well) were incubated with seven scalar doses of lectin or SNLRP (between 2×10^{-13} and 2×10^{-6} M) for 18 h, followed by a 2 h pulse with L-[4,5-^{3}H]leucine (125 nCi/0.25 ml, specific activity 85 Ci/mmol).

IC_{50} (concentration causing 50% inhibition) was calculated by linear regression analysis.

2.4. N-glycosidase activity

N-glycosidase activity was assayed by measuring adenine released from different substrates (ribosomes, DNA, poly(A), rRNA). Ribosomes prepared from a rabbit reticulocyte lysate as described in [26] were a generous gift from Dr. M. Brigotti. Poly(A) and rRNA from *E. coli* (16S+23S) were from Boehringer GmbH (Mannheim, Germany). DNA from herring sperm (hsDNA from Sigma Chemical Co., St. Louis, MO) was treated for 2.5 h at 37°C with DNase-free RNase A (Boehringer), then it was repeatedly precipitated in ethanol to remove the enzyme [6].

Reaction mixtures contained, in a final volume of 50 μl, 50 mM sodium acetate buffer (pH 4.0), 100 mM KCl, 19 pmol ribosomes or 20 μg of polynucleotide substrates, and 5 μg of protein to be tested. After a 40 min incubation at 30°C, the reaction was arrested and adenine released was determined by HPLC as described previously [6]. When indicated, the proteins were reduced as for protein synthesis determination.

2.5. Binding to, uptake and degradation by cells

IRA was labelled with ^{125}I by the Iodogen reagent as described [27].

The day before the experiments cells were trypsinised and seeded in 24-well trays. Binding to, and uptake and degradation by HeLa cells were determined as described previously [28] with minor modifications. Briefly, the cells (0.5×10^5) were incubated at 37°C for the indicated time in 0.5 ml of Hepes-buffered complete medium with 10^{-8} M ^{125}I-labelled lectin ($1.30\pm0.19\times10^5$ cpm/5 pmol). The uptake was arrested by moving the culture to ice. The medium was removed, adjusted to 10% trichloroacetic acid (w/v, final concentration) and centrifuged to determine the acid-soluble radioactivity as a measure of the degradation and exocytosis of degraded lectin. 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), containing 0.04 M NaCl, to quantitate the membrane-bound IRA. The cells were then washed once with phosphate-buffered saline (pH 7.5), and were extracted at 37°C for 10 min with 0.1 M KOH to measure the intracellular accumulation of lectin. 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 internalisation of IRA was calculated as the sum of the intracellular and degraded protein.

The concentration of nucleic acid was measured from the A_{260} corrected for protein concentration by the formula: $62.9\times A_{260}-36\times A_{280}$. The values obtained were compared by ANOVA test [29].

3. Results

3.1. Protein synthesis

Among tested proteins, IRA and SNLRP inhibited cell-free protein synthesis with IC_{50}s below 20 nM, i.e. within the range observed with other RIP (Table 1). In the case of IRA the inhibition was 10 times stronger when the lectin was reduced prior to assay. APA and SNAI had a 20–130

Table 1
Effect of lectins and SNLRP on protein synthesis

	APA	BDA	GNA	IRA	SNAI	SNLRP
Cell-free protein synthesis IC_{50}[a] (nM)						
Reduction						
–	192	>1500	>1500	18.3	370	6.03
+	111	>1500	>1500	1.67	225	5.74
Protein synthesis by cell lines[b] IC_{50}[a] (nM)						
BeWo	>200	n.d.[c]	n.d.[c]	12.7 ± 1.00	72.9 ± 14.2	409 ± 79.4
HeLa	>200	n.d.[c]	n.d.[c]	134 ± 2.67	>400	>1500
JM	>200	n.d.[c]	n.d.[c]	89.3 ± 18.7	>400	863 ± 393
3T3	>200	n.d.[c]	n.d.[c]	10.0 ± 8.33	>400	84.0 ± 9.56

[a]IC_{50}: concentration of lectins or SNLRP inhibiting protein synthesis by 50% as compared to controls calculated by linear regression analysis ($R \pm SD = 0.99 \pm 0.01$ and 0.95 ± 0.05 for cell-free and for cellular protein synthesis, respectively).
[b]Results are mean values ± SD of two experiments performed in triplicate. Incorporation of [^{3}H]leucine by cells in the absence of lectins or SNLRP (controls) was (dpm ± SD): BeWo 16 343 ± 2548, HeLa 21 102 ± 199, JM 9895 ± 2239, and 3T3 3606 ± 1071.
[c]n.d.: not determined.

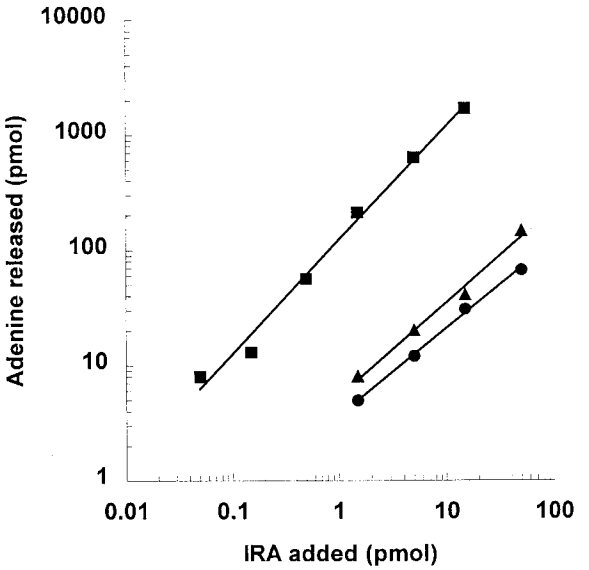

Fig. 1. Dose-response curves of adenine released by *Iris* hybrid agglutinin from various polynucleotidic substrates. The indicated amounts of unreduced *Iris* hybrid agglutinin were incubated at 30°C for 40 min with 20 μg of either (■) hsDNA, (▲) poly(A), or (●) rRNA. Controls were run with complete assay components but agglutinin (<6 pmol adenine were spontaneously released from all substrates). Adenine released (pmol/min/pmol of enzyme) was calculated from linear regression analysis and was: 3.11 from hsDNA, 0.16 from poly(A) and 0.08 from rRNA.

times lower inhibitory activity, BDA and GNA were not inhibitory at the highest concentration tested.

The proteins with inhibitory activity on cell-free protein synthesis were tested for inhibition of protein synthesis by cells. Consistently with the effects on cell-free protein synthesis, IRA was the most active lectin, especially on 3T3 and BeWo cells, followed by SNLRP to which, again, 3T3 fibroblasts were the most sensitive. SNAI inhibited protein synthesis by BeWo cells only, and APA had no effect up to 200 nM (Table 1). It should be noted that the inhibitory effect was different on various cell lines, HeLa cells being the most resistant. In all cases the inhibition was much less than that exerted by ricin, which inhibits cellular protein synthesis with IC_{50} several orders of magnitude lower [2].

3.2. *N*-glycosidase activity

All proteins active on protein synthesis had *N*-glycosidase activity, releasing less than one residue of adenine per ribosome, whereas GNA, which does not affect protein synthesis, had no effect on ribosomes. When tested on substrates other than ribosomes, only IRA and SNLRP had a marked *N*-glycosidase activity, which was greater on hsDNA than on rRNA and poly(A) (Table 2). As for cell protein synthesis, SNAI showed some activity, whereas APA and BDA had only traces of activity. It should be noted that the activity on DNA was generally decreased after reduction of the proteins. Dose-response curves of *Iris* hybrid agglutinin on various polynucleotidic substrates (Fig. 1) showed a linear ratio between enzyme concentration and adenine released.

3.3. Binding to, uptake and degradation by cells

The endocytosis of IRA, the protein with the highest RIP and *N*-glycosidase activities, was studied with HeLa cells (Fig. 2). A significant two-factor interaction was found between binding, intracellular accumulation, degradation and total internalisation, versus the variable time by ANOVA ($F=3.49$; df 15/72; $P=0.0002$). The protein bound was almost undetectable, and the amount internalised by the cells was very small (3.7 pmol/mg nucleic acid after 6 h incubation) as compared with ricin (45.5 pmol/mg nucleic acid) and also lower than the corresponding amount of the non-toxic type 2 RIP nigrin b (53.2 pmol/mg nucleic acid) [30]. Interestingly, the intracellular accumulation of IRA (2.4 pmol/mg nucleic acid) was similar to that of nigrin b (1.9 pmol/mg nucleic acid), and both values were lower than that of ricin (9.6 pmol/mg nucleic acid [30].

4. Discussion

The inhibition of protein synthesis by the active proteins can be attributed to their *N*-glycosidase activity. The proteins even at high concentration removed no more than a single adenine molecule per ribosome, as most RIP do, presumably at the ricin/sarcin site. Moreover, like all RIP tested so far [8], they acted also on hsDNA, which appeared to be the best among non-ribosomal substrate. The polynucleotide:adenosine glycosidase activity of the proteins reflected the effect on cell-free protein synthesis, IRA and SNLRP being the most active and the only ones with some activity also on poly(A) and rRNA as substrates. The activity of SNAI and APA was scarce and limited to DNA, whilst BDA was inactive. The reduced proteins were less active on DNA, as it was observed with ricin [8], whereas their effect on protein syn-

Table 2
N-glycosidase activity

	Adenine released (pmol) from:						
	Ribosomes	hsDNA		poly(A)		rRNA	
Reduction	(+)	(−)	(+)	(−)	(+)	(−)	(+)
APA	8.44	37	25	0	0	1	1
BDA	n.d.	4	4	1	0	4	3
IRA	8.63	6894	4505	212	271	99	125
SNAI	9.27	213	129	3	0	3	2
SNLRP	9.04	2147	1144	25	11	47	42

Lectins or SNLRP (5 μg) were incubated at 30°C for 40 min with 19 pmol of rabbit reticulocyte ribosomes or 20 μg of polynucleotides under the experimental conditions described in the text.
Values obtained in the absence of lectins or SNLRP (controls) were subtracted, and were 4.9 (ribosomes), 6.9 (hsDNA), 6.0 (poly(A)) and 0 (rRNA) pmol of adenine.
Results are mean of duplicate determinations. n.d.: not determined.

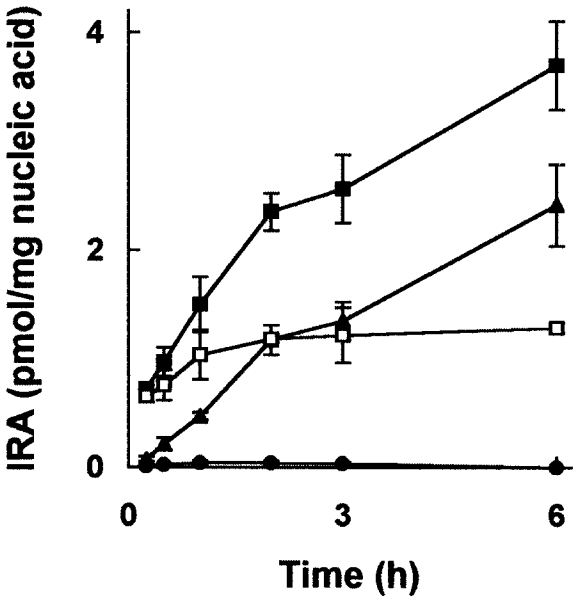

Fig. 2. Internalisation of *Iris* hybrid agglutinin by HeLa cells. Cells were incubated for the indicated time, with 10^{-8} M ^{125}I-labelled lectin. Binding to (●), and intracellular accumulation (▲) and degradation by (□), HeLa cells of IRA were measured as described [28]. The internalisation (■) was calculated as the sum of the intracellular and degraded protein. Results are given (means ± SE) of four experiments in triplicate samples.

thesis was enhanced after reduction. The different steric hindrance of ribosomes and hsDNA may account for this difference, since the disulfide bond is not involved in the active site configuration, as judged from crystallographic studies on RIP (reviewed in [2]).

Consistently with these results, IRA and SNLRP affected protein synthesis by cells, again with IC_{50}s much lower than ricin and related toxins [2]. The chorioncarcinoma-derived BeWo cell line was very sensitive to these proteins, consistently with previous results [31]. This high sensitivity of BeWo cells probably explains why these cells were the only ones inhibited by SNAl, in spite of the low activity of this lectin on the cell-free system. The marked inhibition of 3T3 cell line protein synthesis was unexpected, since fibroblastic cells were generally rather resistant to other RIP [2]. As a whole, these results confirm the different sensitivity to RIP observed with various cell lines [2] and seem to indicate that the action of type 2 RIP on cells depends on the characteristics both of the cells and of the RIP.

5. Conclusion

(i) IRA can be considered as a non-toxic type 2 RIP like nigrin b [30] and cinnamomin [16], on the basis of its structure and of the effect on cell-free and cell protein synthesis, and of its polynucleotide:adenosine glycosidase activity. The inhibition of protein synthesis by cells caused by this lectin, and consequently its cytotoxicity, are lower as compared with ricin. This may be accounted for by the extremely low binding of IRA to the cells, inferior to that of nigrin b which in turn is lower than that of ricin and similar toxic type 2 RIP. Moreover, only 65% of the bound lectin accumulated inside the cytoplasm after internalization, the remaining following a pathway leading to degradation and extrusion from the cells.

(ii) SNLRP also strongly resembles type 2 RIP as far as the enzymatic activity is concerned, but differs in that the B-chain lacks a sugar binding site, and this accounts for the low cytotoxicity. Its inhibitory activity on cell-free protein is not affected by reduction, possibly because the B-chain does not hinder the active site of the A-chain.

(iii) The action of SNAl and APA is not clear, since these lectins have a low enzymatic activity, possible due to their polymeric structure. Furthermore, SNAl inhibits protein synthesis by BeWo cells only.

(iv) It is confirmed that BDA [19] has no RIP activity and, consistently, no polynucleotide:adenosine glycosidase activity. As expected, GNA, which is totally unrelated to RIP, has no enzymatic activity.

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