Cell. Mol. Life Sci. 63 (2006) 1850–1866
1420-682X/06/161850-17
DOI 10.1007/s00018-006-6078-7

Cellular and Molecular Life Sciences

Review

Ribosome-inactivating proteins: progress and problems

F. Stirpe* and M. G. Battelli

Dipartimento di Patologia sperimentale, Alma Mater Studiorum Università di Bologna, Via S. Giacomo 14, 40126 Bologna (Italy), Fax: +39 051 2094746, e-mail: fiorenzo.stirpe@unibo.it

Received 17 February 2006; received after revision 23 March 2006; accepted 2 May 2006
Online First 24 June 2006

Abstract. Ribosome-inactivating proteins (RIPs), mostly from plants, are enzymes which depurinate rRNA, thus inhibiting protein synthesis. They also depurinate other polynucleotide substrates. The biological activity of RIPs is not completely clarified, and sometimes independent of the inhibition of protein synthesis. There are differences in the cytotoxicity of RIPs and, consequently, in their toxicity to animals. Some RIPs are potent toxins, the best known being ricin, a potential biological weapon. New toxins have recently been identified. RIPs cause apoptotic and necrotic lesions, and induce production of cytokines causing inflammation. RIPs are potentially useful in agriculture and medicine because (i) they have antiviral activity and (ii) they are used for the preparation of conjugates with antibodies ('immunotoxins') or other carriers, rendering them specifically toxic to the cell target of the carrier, which may be helpful in therapy. The distribution, mechanism of action and role in nature of RIPs are not completely understood, and we can expect several future developments in their practical application.

Keywords. Ribosome-inactivating protein, toxin, lectin, N-glycosylase, immunotoxin.

Ribosome-inactivating proteins (RIPs) are enzymes which damage ribosomes in an irreversible manner by removing one or more adenine residues from rRNA; they also depurinate other polynucleotides. The history of ricin and related toxins was well reviewed recently by Olsnes [1]. There are a number of recent reviews on the subject [2–7], so the present one will deal with the main biological properties of RIPs, focusing on unclear, unresolved and/or controversial issues, covering the most recent advances.

Nomenclature and classification

Two potent toxins, ricin, from the seeds of *Ricinus communis*, and abrin, from the seeds of *Abrus precatorius*, were known at the end of the 19th century, and were used by Paul Ehrlich to raise the first antibodies. Seventy years later, when their structure and mode of action were elucidated, these toxins turned out to be the very first identified RIPs. Ricin and abrin were found to be composed of two polypeptide chains, an enzymatic A chain that damaged ribosomes, and a lectinic B chain, galactose specific, capable of binding to cells [8, 9]. Other and more numerous proteins were found that resemble the A chains of these toxins, in that they have a similar structure and the same activity on ribosomes. The structure [10] and genetics [11, 12] of RIPs have been exhaustively reviewed.

These proteins were called RIPs [13], because they were found to damage ribosomes catalytically, i.e. enzymatically, and the designation was intended to be provisional, until the exact nature of the enzymatic activity of the proteins was known.

The proposal was made to divide the RIPs into type 1, single-chain, strongly basic proteins of approximately 30 kDa with enzymic activity, and type 2, consisting of two chains, one of approximately 30 kDa with enzymic activity, and one of approximately 35 kDa with lectin properties. Subsequently, a type 3 was proposed, comprising a maize protein b-32, which becomes active only after the removal of a short internal peptide segment [14], and a barley RIP,

* Corresponding author.

Table 1. Toxic type 2 RIPs (toxins).

Toxin	Source	Toxicity	
		to HeLa cells IC_{50} [a] (M)	to mice LD_{50} [b] (μg/kg)
Abrin	*Abrus precatorius* seeds	3.9×10^{-12} [20]	2.8 [9]
Ricin	*Ricinus communis* seeds	6×10^{-13} [21]	8 [8]
Mistletoe lectin I	*Viscum album* leaves	1.7×10^{-9} [20]	2.4 [22]
Modeccin	*Adenia digitata* root	2.8×10^{-12} [20]	5.3 [23]
Volkensin	*Adenia volkensii* root	3×10^{-13} [24]	1.7 [24]
RIP	*Adenia goetzii* caudex	1×10^{-12} [25]	
Lanceolin	*Adenia lanceolata* caudex	5×10^{-13} [25]	6.8 [c]
Stenodactylin	*Adenia stenodactyla* caudex	3×10^{-13} [25]	< 1.2 [c]
Aralin	*Aralia elata* shoots	1.3×10^{-12} [d] [26]	
Riproximin	*Ximenia americana* powder [27]	1.1×10^{-12} [e]	

[a] Concentration causing 50% inhibition of protein synthesis.
[b] Dose killing 50% of the animals within 7 days.
[c] Unpublished results from our laboratory.
[d] Dose killing 50% of the cells.
[e] Personal communication from C. Voss.

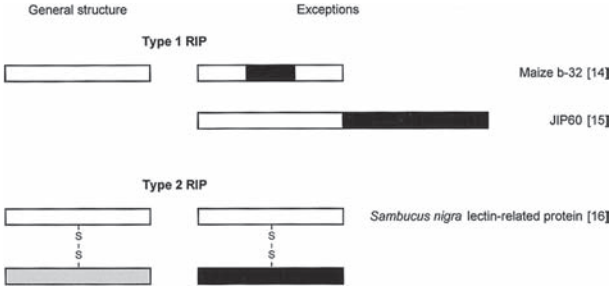

Figure 1. Structure of type 1 and 2 RIPs: active chain (open bar), binding chain (black bar) and chain with unknown function (grey bar).

JIP60, in which the active chain is linked to a segment of similar size with unknown function [15], or only the barley RIP, according to Peumans et al. [3]. Also proposed were holo-RIPs, for those with a single chain or two smaller polypeptide chains (type 1 RIPs) and chimero-RIPs, for two-chain proteins (type 2 and the single type 3 RIPs) [4]. As stated in a previous review [7], we prefer to maintain the old RIP nomenclature, which is based on the absence (type 1 RIPs) or presence (type 2 RIPs) of a lectinic chain, considering as exceptions to type 1 those RIPs with an additional segment [14, 15], and to type 2, the RIP from *Sambucus nigra* with a non-lectinic B chain [16] (Fig. 1). Some type 2 RIPs are potent toxins, while others are much less toxic (LD_{50} for mice in the order of μg/kg and mg/kg, respectively). The reasons for this difference are still not completely known, and will be discussed below.

Distribution in nature

RIPs are widespread and almost ubiquitous among plants, with type 1 RIPs, in particular, being found at various concentrations in many plants, including some that are eaten raw (e.g. spinach [17, 18], tomato [19]). More type 1 than type 2 RIPs have been described, and only a few highly toxic type 2 RIPs are known.

Several reviews [4, 5, 7] provide lists of RIPs, and relevant references can be found in Girbés et al. [5]. Recent findings of new toxic type 2 RIPs are noteworthy and an up-to-date list of these toxins is given in Table 1. To help the reader, a list of all other RIPs mentioned in this review and of their sources is given in Table 2.

A toxic type 2 RIP, aralin, was found in the shoots of *Aralia elata* [26], which are described as edible and presumably can be eaten safely because the RIP is destroyed by cooking and its concentrations probably too low (0.32 mg/100 g) to be harmful when taken by the oral route. The latter observation is of general interest, because it suggests that other unknown toxic type 2 RIPs may be present in other, possibly many, plants, which have not been examined because they are non-toxic.

Another protein, toxic at least to cells, was found in *Ximenia americana* and seems to be a type 2 RIP [27]. Other highly toxic type 2 RIPs were found in three Passifloraceae, *Adenia goetzii*, *Adenia lanceolata* and *Adenia stenodactyla* [25]. Finding these toxins was not surprising, since two type 2 RIPs, modeccin and volkensin, had been found previously in *Adenia* (*Modecca*) *digitata* and *Adenia volkensii*, respectively. Thus, type 2 RIPs seem to be particularly frequent among Adenia plants, many of which (all those examined) contain galactose-binding lectins [25].

RIPs may be present in one or more tissues of a plant, sometimes in more than one form: ricin is present only in the seeds of *Ricinus* plants, whereas saporin is present in several forms in all tissues of soapwort [28]. Sometimes,

Table 2. RIPs mentioned in the present review, and their sources.

RIP	Source		
	scientific name	common denomination	tissue
Type 1 RIPs			
Agrostin	*Agrostemma githago*	corn cockle	seed
b-32	*Zea mais*	maize	seed
Bouganin	*Bougainvillea spectabilis*		leaf
Camphorin	*Cinnamomum camphora*	camphor tree	seed
Curcin	*Jatropha curcas*		seed
Gelonin	*Gelonium multiflorum*		seed
JIP60	*Hordeum vulgare*	barley	seed
Momordin	*Momordica charantia*	bitter gourd	seed
Pokeweed antiviral protein (PAP)	*Phytolacca americana*	pokeweed	leaf, seed, root
Saporin	*Saponaria officinalis*	soapwort	seed, leaf, root
Trichosanthin	*Trichosanthes kirilowii*		tuber
Non-toxic type 2 RIPs			
Ebulin	*Sambucus ebulus*		leaf, fruit
Porrectin	*Cinnamomum porrectum*		seed

both type 1 and type 2 RIPs have been found in the same plant [e.g. *Sambucus* spp., reviewed in ref. 29], *Cinnamomum camphora* [30], *Iris hollandica* [31], and toxic and non-toxic RIPs may coexist (e.g. ricin and *Ricinus* agglutinin in castor beans). RIPs are produced also by calluses [18, 32] and plant cells in culture [reviewed in ref. 5], by bacteria (Shiga and Shiga-like toxins) [reviewed in ref. 33], by an alga, *Laminaria japonica*, and by mushrooms [reviewed in refs. 5, 7]. An RIP-like glycosylase activity (see later) was also found in mammalian cells and tissues [34], which was scarce and very labile, and defied all attempts at purification.

In plants, the expression of RIPs is enhanced in senescence [35–37] and in various unfavourable conditions, such as various types of stress [15, 36, 37], viral infection [38, 39] and contamination by microorganisms [40]. It is noteworthy that JIP60 [35] and curcin [41], two type 1 RIPs from barley and *Jatropha curcas*, respectively, are detectable only in conditions of stress and that the RIP-like glycosylase activity of mammalian cells is higher in stressed and poliovirus-infected cells [34]. An RIP, BP31, is also present in spinach calluses during the embryogenesis induced by gibberellic acid [18], a condition considered as triggered by stress by the authors. All this may have implications for understanding the role of RIPs in nature.

Enzymatic activity

Early biochemical studies on ricin revealed that the toxin inhibited protein synthesis in cells and in cell-free systems [reviewed in ref. 1], and the latter effect led to the identification of the target of ricin as the ribosomes [42], which were rendered unable to bind elongation factor 2 [43]. Endo and colleagues discovered the N-glycosidase activity of the toxin, consisting in the removal of a single adenine residue (A_{4324} in rat liver rRNA) from a GAGA sequence in a universally conserved loop at the top of a stem in 28S rRNA, the so-called sarcin/ricin domain [44] (Fig. 2). The research was extended to all RIPs, which all had a similar activity and were officially classified as rRNA N-glycosidases (EC 3.2.2.22). RIPs access ribosomes by binding to ribosomal proteins: saporin to an unidentified 30-kDa protein [46] which, interestingly, is not present in *Escherichia coli* ribosomes, less sensitive to saporin, ricin A chain to L9 and L10e proteins [47], pokeweed antiviral protein (PAP) to L3 [48]. PAP binds also to the initiation factors 4G and iso4G [49] and this

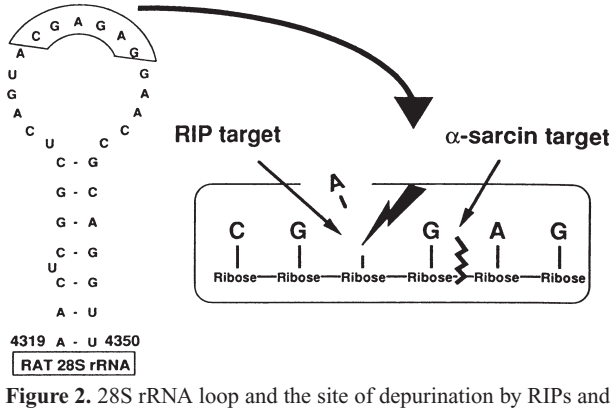

Figure 2. 28S rRNA loop and the site of depurination by RIPs and α-sarcin [from ref. 45].

may have implications for the depurination of viral RNAs. Trichosanthin binds to L10a [50], P0 and P1 [51], which interferes with the binding of the elongation factors, and also to a MAD2B protein that may be involved in cell cycle signalling [51], with which, consequently, this RIP might interfere.

There are differences between the various RIPs in their action on ribosomes. Thus, some, but not all RIPs are active only in the presence of ATP and supernatant cofactors [52]. Both ATP and cofactors act on ribosomes rather than on the RIPs and some cofactors have been identified as tRNAs, which were different for various RIPs ($tRNA^{Trp}$ for gelonin [53], $tRNA^{Ala}$ for agrostin, $tRNA^{Ala}$ and $tRNA^{Val}$ for barley RIP, and $tRNA^{Gly}$ for PAP-S [54]).

Subsequently, RIPs were found to remove adenine from various polynucleotides other than rRNA [55] and, consequently, the denomination of adenine polynucleotide glycosylase was suggested as more appropriate. However, as stated by Hartley and Lord [6], it is difficult to reconcile with other findings, since there are no reports of kinetics with various substrates.There is also evidence that the effect on protein synthesis cannot account for the cytotoxic properties of RIPs, which must exert some other action(s) (see below).

More recently [56], RIPs were observed to remove adenine from the ADP-ribose chain of activated poly(ADP-ribose) polymerase (PARP), which is involved in the mechanism of DNA repair. This damage to activated PARP may have a role in the inhibition of DNA repair by RIPs, which seems to be independent of the inhibition of protein synthesis [57]. As a whole, these alterations may be involved in the transforming activity of RIPs that has also been observed [56].

Furthermore, both Shiga toxin and ricin have been reported to cause early nuclear DNA damage in endothelial cells, concomitant with (ricin) or after (Shiga toxin) inhibition of protein synthesis and well before the onset of apoptosis, indicated by the activation of caspases [58]. Interestingly, inhibition of translation by cycloheximide did not cause a similar alteration. These results indicate the occurrence of an early direct damage to DNA, independent of the inhibition of protein synthesis, and suggest that these toxins may enter the nucleus.

The question of other enzyme activities of RIPs is still unclear. RIPs have been suggested to possess nuclease activities [exhaustively reviewed in ref. 3]. Several authors have reported various activities of this type, usually observed with high concentrations of RIPs [reviewed in ref. 59]. In some cases, the nuclease could be separated from the glycosidase activity [60, 61] and was not detected when RIPs were accurately purified [62]. This issue remains open, and differences in the preparation and assay conditions of RIPs do not help in finding a solution.

A puzzling finding is the superoxide dismutase (SOD) activity of RIPs. Some type 1 RIPs, camphorin, from *C camphora* [63], porrectin, from *C. porrectum* [64], a RIP-type protein from tobacco [65] and a RIP from *Cucurbita moschata* [19] have been reported to have SOD activity at a level that seemed to exclude the possibility of contamination. Purified SOD was also reported to have RIP activity [65] and the tobacco protein had a sequence similar to that of SOD, thus leading to the hypothesis that the two proteins had dual enzymatic activity. An antioxidant activity of RIPs from *Celosia cristata* has also been reported [66].

Toxicity

The toxic effects of some type 2 RIPs, including the toxicity of castor and jequirity beans to animals and humans, as well as the abortifacient activity of some Cucurbitaceae, have been known since ancient times, well before the identification of the proteins responsible for them. Up to the beginning of the 1970s, the toxicity was the only reason for the attention of the scientific community to plant RIPs. Later a number of these proteins was described and classified on the basis of both their structure and toxicity, since the toxicity of type 2 RIPs was often higher than that of type 1 RIPs. Moreover, the toxicity to cells correlates well with the toxicity to animals and humans, although with some exceptions (see Table 1). The mechanism of ricin cytotoxicity has been reviewed recently [67].

Interaction with cells

Consistently with the lower cytotoxicity of type 1 as compared with type 2 RIPs, the entry process appears to depend largely on the protein structure. Thus, the differences observed in the toxicity of different RIPs were ascribed to the absence or presence of B chains with properties of lectins specific for galactose, N-acetyl galactosamine or N-acetyl neuraminic acid [reviewed in ref. 4], which allow binding to the cell membrane of type 2 RIPs and facilitate their endocytosis process [8, 9]. Indeed, the absence of the lectin moiety considerably limits the entry of type 1 RIPs into cells and justifies their low level of toxicity to cells and consequently to animals. However, the presence of the B chain is not sufficient to confer a high level of cytotoxicity on type 2 RIPs (see below). Type 1 RIPs, though, are very toxic if allowed to enter cells in various ways, by enclosure into carriers that can fuse with cells such as liposomes, erythrocyte ghosts or viral envelopes [reviewed in ref. 68], by subjecting cells to shock waves [69], by photochemical internalization [70, 71] or by linkage to appropriate carriers capable of binding to cells (see the paragraph on immunotoxins below).

The endocytosis mechanism of type 1 RIPs is still not well known. The entry of saporin-S6 into many cell types was

shown to be mediated by the α_2-macroglobulin receptor [72] as observed for trichosanthin [73] and *Pseudomonas* exotoxin A [74]. However, the correlation between the level of this receptor and the sensitivity to saporin-S6 is lacking in the case of some cell types, suggesting an α_2-macroglobulin receptor-independent way of saporin-S6 endocytosis [75]. Recently, the intracellular routes followed by saporin and ricin to intoxicated cells were compared [76]. Ricin was confirmed to enter the cytosol after a Golgi-mediated retrograde transport, whereas saporin utilized a Golgi-independent pathway.

On the other hand, a recent study [77] reported that the retro-translocation of PAP from endoplasmic reticulum into cytosol is similar to that utilized by type 2 RIPs to reach their subcellular target. The retro-translocation was described when PAP was expressed by yeast cells however, this observation suggests that type 1 RIPs may also be able to follow the cellular route for misfolded proteins without being degraded by the proteasome. The same authors reported that site-directed mutagenesis of PAP expressed by yeast cells abolished cytotoxicity, although not affecting ribosome depurination, thus indicating that the inhibition of protein synthesis is not sufficient for cytotoxicity [77, 78].

Toxic and non-toxic type 2 RIPs

The two-chain type 2 RIPs have been grossly divided into two groups, toxic and non-toxic, based on the considerable differences in their cytotoxicity, and consequently in their toxicity to animals, which varies by some three orders of magnitude [reviewed in refs. 7, 79]. Differences exist also within the group of toxic RIPs, abrin, volkensin [reviewed in ref. 80] and the toxin from *A. stenodactyla* [25] being more potent than ricin. The reasons for these differences are not completely known and could be many; they most likely involve the binding to, and entry into, cells and/or the intracellular destination, degradation and exocytosis of the proteins.

Differences in the number of receptors do not account for the different cytotoxicities: thus on the surface of HeLa cells, there are more receptors for ricin ($1–3 \times 10^7$) [81] than for the more toxic RIPs modeccin and volkensin (2×10^5) [82, 83].

After the binding of type 2 RIPs to glycoproteins and glycolipids on the cell surface, different endocytosis processes, either clathrin dependent or independent, direct the molecules to early endosomal vesicles where they can be sorted for further routing, depending on the nature of the receptor, and led to various fates [reviewed in ref. 68]. Many studies, mostly conducted with ricin, have described how essential the intracellular localization in the trans-Golgi network (TGN) was to the cytotoxicity of RIPs [reviewed in ref. 84]. The TGN is the cellular compartment in which both the endocytic and secretory pathways converge. Indeed, carrier proteins with a retrieval signal for endoplasmic reticulum (ER) usually recycle between ER and the Golgi apparatus, being transported up to the TGN. It has been proposed that ricin is able to bind to a recycling galactosylated component, because of the requirement for a functional B chain for its cytotoxicity [reviewed in ref. 85]. During the last few years, the availability of retrograde transport has been assessed for toxic proteins, either with or without the retrieval KDEL sequence, through the secretory pathways from the Golgi apparatus to the ER [reviewed in ref. 86].

The RIP molecule or at least its active chain must then enter the cytosol to gain access to the ribosomal substrate. It has been proposed that the two chains of type 2 RIPs are separated in the ER lumen by the protein disulphide isomerase [87]. The translocation of ricin to the cytosol was demonstrated to occur in the ER [88]. The free A chain of type 2 RIPs in the ER lumen could be interpreted as an unassembled polypeptide, sent to the cytosol by the ER quality control system [reviewed in ref. 85]. Indeed, type 2 RIPs are retro-translocated by utilizing the ER-associated degradation pathway usually followed by misfolded proteins, which in the cytosol are polyubiquitinated and degraded by the proteasome [89]. The ability to escape, at least in part, degradation by the proteasome machinery is thus essential for type 2 RIP cytotoxicity [reviewed in ref. 90] (Fig. 3).

The first non-toxic type 2 RIP was identified in castor beans which contain, besides ricin, a much less toxic tetrameric Ricinus agglutinin (RCA) whose A and B chains are similar, but not identical, to the corresponding A and B chains of ricin (94 and 83% homologies, respectively [91]). Both the native [92, 93] and the recombinant [94] A chains of the agglutinin are 15- and 11-fold, respectively, less active in inhibiting cell-free protein synthesis than the A chains of native and recombinant ricin. The reduced toxicity of RCA may be due to its reduced capacity to translocate [95], and that of ebulin, another non-toxic type 2 RIP, from *S. ebulus*, to its reduced affinity for galactose [96]. These differences, however, are of one order of magnitude, approximately, thus not accounting for the much higher toxicity of ricin compared with the agglutinin. Furthermore, ricin is some 68-fold more toxic to cells than RCA [95], but some 2000-fold more toxic to mice [97]. The hypothesis was formulated [98] that, although both lectins can bind to serum glycoproteins and red blood cells, only the agglutinin, being divalent, can form precipitates or agglutinate red cells, thus being prevented from reaching cells in vital organs.

Among other non-toxic type 2 RIPs, the uptake by cells and the intracellular routing and processing of nigrin b, from *S. nigra*, have been studied and this lectin was found to enter cells as well as ricin, but is more rapidly and extensively degraded in, and excreted by, cells [83, 99].

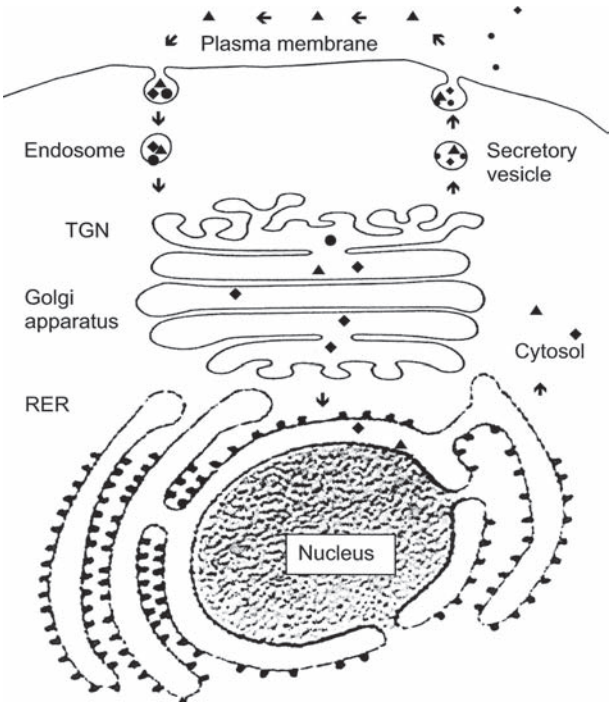

Figure 3. Pathways proposed for intracellular transport of type 2 RIPs: nigrin (●), ricin (♦) and volkensin (▲). Small symbols indicate inactive or degraded molecules.

Volkensin too is rapidly excreted by cells, but mostly in a non-degraded form, thus being available for further entering into cells, which may account at least in part for the higher toxicity of this RIP, compared with ricin [83]. The resistance to degradation of volkensin may be due to its low content of lysine [100], as ricin and abrin, which also have a low lysine content, were reported to be more resistant to degradation when some lysine residues were removed, whereas they were more easily degraded when the lysine content was increased [101]. These results, though, should be considered with some caution, because the intracellular degradation of the toxins may occur through mechanisms quite different from those of the proteolytic enzymes used in *in vitro* experiments.

The studies on the lesions caused in animals by RIPs [reviewed in ref. 79], mostly performed with ricin, raise puzzling considerations. The first is that the various toxins, despite their apparently similar mechanism of action, cause different lesions, e.g. ricin brings about massive necrosis in the intestine of poisoned rats [102] which is not seen in rats poisoned with abrin [103], possibly because of a different distribution within the organism, in turn probably due to differences in the B chains.

The lesions observed in animals poisoned with type 2 RIPs are different from those caused by other inhibitors of protein synthesis, which do not bring about the apoptotic and sometimes massive necrotic lesions observed in the animals poisoned with ricin and volkensin [reviewed in ref. 79] or even with high doses of type 1 RIPs or their immunotoxins [104]. This suggests that RIPs must do something else other than inactivating ribosomes. An important factor is probably the production of cytokines, which may be released by macrophages damaged by RIPs to which they are especially sensitive [reviewed in ref. 68] and may cause apoptosis [reviewed in ref. 105] and inflammatory reactions. Indeed, viscumin [106, 107], ricin [108–113], modeccin [112] and Shiga toxins [reviewed in ref. 114] all induce production of tumour necrosis factor (TNF) and interleukins by human mononuclear cells. The effect of Shiga toxins is particularly interesting, in that they cause release of cytokines, including interleukin-8 from intestinal epithelial cells [115], still maintaining a level of protein synthesis sufficient to ensure their production [116]. Moreover, the production of interleukin-8 was recently observed to be stimulated by ricin, but not by cycloheximide at a concentration causing an equivalent inhibition of protein synthesis [117].

Consistent with the observations on cells, the level of TNF and other cytokines was increased in blood serum of rats [108] and mice [113] poisoned with ricin. The release of these cytokines is a consequence of the 'ribotoxic stress response' [118] with activation of several protein kinases (ERK, JNK and p38 MAPK) described in cells exposed to ricin at concentrations which caused minimal inhibition of protein synthesis [reviewed in ref. 113]. Shiga toxin too activates p38 and JNK kinases in intestinal epithelial cells [119]. These alterations may explain the general inflammation observed in animals poisoned with ricin and other RIPs. It is noteworthy that the inflammatory condition described in rats [120] and mice [113] poisoned with ricin resembles the haemolytic uremic syndrome caused by Shiga toxin in humans.

The expression of interleukins was also induced by the type 1 RIP trichosanthin both *in vitro*, in peritoneal macrophages [121], and *in vivo*, in mice [122].

RIP-induced apoptosis

Starting from the late 1980s, apoptotic cell death caused by type 2 RIPs has been reported *in vivo* and *in vitro*, as well as by free or conjugated type 1 RIPs [reviewed in refs. 59, 79]. Immunotoxins containing saporin and bouganin inhibit cell protein synthesis, induce apoptosis, and block the clonogenic growth of target cells, although with a different kinetics, suggesting the presence of different mechanisms of cell killing [123]. Consistently, the isolated B chain of ricin, although devoid of protein synthesis-inhibiting activity, was able to induce apoptosis, possibly by linking molecules on the cell surface which activate programmed cell death [124]. Besides the 'ribotoxic stress response' mentioned above, multiple apoptotic signalling pathways may, thus, be triggered by intoxication with RIPs. Viscumin induced apoptosis

by decreasing the expression of the anti-apoptotic Mcl-1 and activating the caspase-dependent pathway [125]. Trichosanthin was shown to cause apoptosis via a nitric oxide (NO)-mediated pathway [126] and also by inducing the production of reactive oxygen species [127]. Ricin A chain-induced apoptosis appeared to be independent of the inhibition of protein synthesis and to be linked to a structural motif in the RIP molecule which is far removed from the catalytic site [128]. This sequence could be responsible for the caspase 3-mediated apoptotic action of ricin A chain or ricin A chain-containing immunotoxins on endothelial cells [128]. This apoptotic pathway was induced by abrin too and involves mitochondrial membrane potential damage and reactive oxygen species production [129], consistently with the observation that abrin inactivates a thiol-specific antioxidant protein [130]. Ricin-induced NAD^+ and ATP depletion, which can be prevented by the inhibition of poly(ADP-ribose) polymerase, was also reported to cause apoptosis [131]. Finally, ricin was shown to damage nuclear DNA in whole cells by means that do not appear to be secondary to ribosome inactivation, suggesting a mechanism of apoptosis centred on the enzymatically induced DNA lesion [58]. Again, the behaviour of Shiga toxins is peculiar in that they seem to downregulate antiapoptotic [132] and enhance proapoptotic [133] factors.

Retrograde transport in the nervous system

All toxic type 2 RIPs share with other lectins the property of being retrogradely transported when injected into peripheral nerves [reviewed in refs. 134, 135]. By microinjecting ricin into nerves, this 'suicide transport' has been exploited to ablate motor and sensory neurons projecting through the injected nerve. Only modeccin and volkensin are transported retrogradely when injected in the central nervous system, presumably due to different properties of their B chains. These were the first agents producing efficient destruction of neurons afferent to the injection site in the central nervous system and have been used to study a variety of projections and to produce selective lesions in the central nervous system [134]. The retrograde transport of toxins has been suggested for the selective destruction of neuromas [reviewed in ref. 136].

Allergies

Allergy to castor beans is well known [137], and seems to be due to a small allergen [138]. However, the same group showed that ricin enhances the IgE response to ovalbumin in rats [139] and there is ample evidence that many and possibly all RIPs are allergenic. Indeed, allergic symptoms were observed in women receiving trichosanthin, a type 1 RIP from *Trichosanthes kirilowii* used to induce abortion [140], as well as in patients treated with RIP-containing immunotoxins [reviewed in ref. 141], and among personnel working with RIPs in a research laboratory [142]. Allergy to elderberry has also been attributed to a protein with the characteristics of a type 1 RIP [143].
Since RIPs are present in plants that are eaten raw [5, 19], their allergenic properties may have a role in the pathogenesis of the allergies caused by some vegetables.

Antiviral and other antiparasitic activities

All type 1 RIPs tested so far have antiviral activity against plant, fungal and animal viruses [reviewed in refs. 144, 145], whereas only a few type 2 RIPs were found to be active (e.g. those from *Eranthis hyemalis* [146] and some from *S. nigra* [147]).
The effect of RIPs on plant viruses, the first to be known, led to transfection of plants with RIP genes, to improve their resistance to viral infections. Indeed, some protection from some viruses was conferred to transfected plants by several type 1 RIPs [reviewed in refs. 144, 145] and, although with less efficiency, by type 2 RIPs from *S. nigra*, SNA-I' [147] and SNA-V [148], but not by SNA-If from the same plant [149]. High-level expression of PAP was harmful to transfected tobacco [150] and bentgrass [151] plants. This, however, could be prevented by transfecting tobacco plants with a truncated form of ribosomal protein L3, to which PAP binds [152].
The antiviral activity against animal viruses has led to numerous studies on the effect of RIPs, especially PAP and trichosanthin, in HIV-infected cells. The replication of HIV in cells was inhibited by several RIPs [reviewed in ref. 145], and investigations were started with the hope that RIPs could be used in the therapy of AIDS [reviewed in ref. 153]. Unfortunately, besides the obstacle of the immune response against these foreign proteins, the few clinical trials made with trichosanthin gave disappointing results, and sometimes the administered protein aggravated the neurological [154, 155] or mental symptoms [156] and caused allergic reactions [156].
The mechanism through which RIPs exert their antiviral activity is still not clear. Initially it was thought that RIPs could come in contact with, and damage ribosomes of the infected cells, with consequent death of the cells and arrest of viral proliferation. However, with the advent of recombinant techniques which allowed the production of mutated RIPs, it was possible to ascertain that the antiviral and ribosome-inactivating activities could be separated. Thus TAP29 (actually trichosanthin) inhibited HIV at a concentration that had little effect on ribosomes [157]. A PAP mutant devoid of the C terminus did not depurinate ribosomes, and yet was still able to prevent virus infection in tobacco plants [158, 159] whereas, conversely,

mutants of trichosanthin which had lost most of their anti-HIV activity still retained full N-glycosidase activity [160]. Together, these observations suggest a mechanism different from the glycosidase activity for the antiviral effect of RIPs. Since PAP depurinates HIV RNA [161], RIPs may act directly on viral or virally induced nucleic acids. Furthermore, the anti-HIV activity of trichosanthin was recently reported to be counteracted by an inhibitor of c-Jun N-terminal kinases [162], which indicates an important role of these enzymes in the antiviral activity of trichosanthin and possibly of other RIPs. Finally, RIPs may induce increased resistance to viruses in an indirect way, by stimulating other plant defence systems.

RIPs have also antibacterial, antifungal and insecticidal activities [reviewed in ref. 2], although apparently conflicting results have been reported. Thus the induced expression of a RIP from *Phytolacca heterotepala* enhanced resistance of tobacco plants to the fungi *Alternaria alternata* and *Botrytis cinerea* [163], whereas transfection of *Vitis vinifera* plants with barley RIP did not improve resistance to *Uncinula necator* and *Plasmopara viticola* [164]. There are possibly differences both in the activity of the various RIPs and the sensitivity of fungal species.

Abortifacient activity

The extract of the roots of the Cucurbitacea *T. kirilowii* have been used in Chinese traditional medicine to induce abortion, an effect due to trichosanthin, a protein used as abortifacient in official Chinese medicine [reviewed in ref. 165]. Trichosanthin is an RIP and, conversely, other RIPs examined have abortifacient activity [166], a property subsequently found to be common to all RIPs tested [reviewed in ref. 167]. Actually, RIPs are not abortifacient in the classical sense, in that they do not induce abortion by causing contractions of the uterus or a hormonal imbalance: rather, they cause the death of the fetus by killing syncytiotrophoblasts. These cells are highly sensitive to RIPs [168], presumably because, like macrophages, they have a high capacity of protein uptake, thus taking up a large amount of RIP. The abortifacient activity of trichosanthin was attributed to its effect on ribosomes. However, this may not be the only mechanism, as discussed above in the case of the antiviral activity of RIPs.

Anticancer activity

The use of type 2 toxic RIPs as anticancer agents has been investigated. Extracts of *Viscum album* (mistletoe) and *X. americana*, both containing type 2 RIPs (Table 1), have been used for this purpose for some time in Europe and Africa, respectively. Research on ricin and subsequently on related toxins was stimulated by the higher toxicity of ricin and abrin to cancerous as compared to non-cancerous cells [169]. It is possible that these and other RIPs are more harmful to malignant cells because they have a high rate of protein synthesis while actively proliferating, and also because, being altered, may be more sensitive to the toxins. The alleged antitumour activity of mistletoe extracts is attributed to the type 2 toxic RIP viscumin, and *X. americana* extracts used in Africa to treat cancer contain a type 2 toxic RIP called riproximin [27]. These and possibly other RIPs may have some beneficial effect in cancer patients, not only by acting directly on cancer cells, but also by exerting strong stimulation of the immune system and inducing the production of cytokines, as seen in cells under the effect of several RIPs [112] and also in volunteers receiving mistletoe extracts [170].

Immunotoxins and other conjugates

RIPs have been chemically linked to, or genetically fused with, carriers capable of delivering them to a given type of cell in a selective manner. Mainly antibodies, but also growth factors, hormones and lectins have been used. Type 2 RIPs are not suitable for this purpose, because their B chains can bind to virtually any cell anyway. Thus type 1 RIPs and isolated A chains of type 2 RIPs have been used. The subject has been dealt with in a number of recent reviews [171–174] and here only the possible applications and problems will be discussed.

Although various applications have been envisaged (Table 3), most research on immunotoxins has focused on their possible use in the therapy of cancer. Promising and sometimes impressive results have been obtained, especially on tumours transplanted in animals, and also in patients with various forms of cancer, especially of haematological origin [reviewed in ref. 176], but much less in solid tumours, possibly due to poor penetration. Compared with conventional chemotherapeutic agents, RIP-containing immunotoxins should have some advantages, being very potent, acting on both dividing and non-dividing cells, and not inducing resistance. However, so

Table 3. Possible applications of immunotoxins and other conjugates.

Removal of cells from cultures [175]
Tumour therapy
Haematological malignancies [176]
Solid tumours, [177] including brain tumours [178]
Bladder tumours [179, 180]
Anti-inflammatory (removal of macrophages) [181–183]
Anti-spasm (destruction of muscles) [reviewed in ref. 173]
Ophthalmology
Corneal opacification [184]
Strabismus [185]
Pain killing (destruction of sensitive nerves) [186]

Table 4. Side-effects of immunotoxins made with RIPs [172].

Formation of antibodies against the antibody and the RIP, with consequent allergic reactions, and even anaphylactic shock
Capillary leak syndrome
Hepatotoxicity
Renal insufficiency
Fatigue; myalgia
Fever

far, the use of immunotoxins in cancer therapy has not fulfilled the hopes, undoubtedly excessive, which were raised when they came out. The main difficulties are summarized in Table 4. It should be possible, though, to circumvent or reduce at least some of these problems. Thus the immune response against the antibody could be avoided with the use of humanized and ultimately human antibodies and the consequences of the response against the RIP could be overcome with cycles of therapy with a rotation of immunologically different RIPs. A saporin-containing immunotoxin could be safely administered to severely immunodeficient patients [187]. The use of the presumably less immunogenic RIP-like protein identified in human cells [34] could be considered, if it is isolated and characterized. The vascular leak syndrome could be reduced, if not avoided, by careful dosage and administration of steroids [reviewed in ref. 141].

Many investigators think that immunotoxins could be useful for the therapy of minimal residual disease. So far, immunotoxins have been administered only to patients at an advanced stage, i.e. with large tumour masses, which were sometimes reduced. Consequently, small clumps of, or even isolated tumour cells remaining after conventional treatments might be completely removed by a short-term administration of immunotoxin. Furthermore, various applications have been envisaged which could be more practicable than the use of systemically administered immunotoxins. A possibility is the use of immunotoxins administered intravesically for the therapy of bladder cancer. In this way, immunotoxins would be 'external' to the organism with the consequences that (i) they would probably not cause an immune response and (ii) their toxicity would be minimal, thus allowing the use of relatively high concentrations on cancer cells. RIP-containing immunotoxins [188, 189] and a fibroblast growth factor-saporin conjugate [190] specific for bladder tumour cells have been prepared, and clinical trials with immunotoxins have been performed, with encouraging results [179, 180].

Muscle-specific immunotoxins are potential immunotherapeutic agents for the treatment of focal muscle spasm [191], myasthenia gravis [192] and strabismus, by destroying oculomotor muscles [185, 193–195]. Other possible uses of immunotoxins in ophthalmology have been explored, for the treatment of posterior capsule opacification, for which some clinical trials have been performed [184, 196], and to prevent corneal cell proliferation [197].

A number of interesting results were obtained with the use of saporin-containing immunotoxins and other conjugates directed against a variety of nervous cells. This 'molecular neurosurgery' has been extensively dealt with in various recent reviews [136, 198] and books [134, 199]. Among the main results, a model of Alzheimer's disease was obtained by selective destruction of the basal forebrain level [200]. Also, saporin-substance P conjugates have been used to selectively destroy sensory neurons [201; reviewed in ref. 136], and their potential use for treating patients with chronic dehabilitating pain has been postulated [186].

Further applications of RIPs include the use of B chains in the preparation of conjugates. The intracellular transport of molecules has been obtained with an entirely different, almost reverse approach, with ricin B chain as a carrier of antigens [202, 203]. Furthermore, a fusion protein combining the endotoxin of *Bacillus thuringiensis* with the ricin B chain was prepared, and transgenic rice and maize plants expressing the fusion protein were more toxic to insects than plants containing the toxin gene alone [204].

Bioweapons

Toxic type 2 RIPs are potential biohazards, and from time to time there are reports of accidents due to ingestion of toxin-containing seeds or roots causing intoxications which cannot be effectively treated. Furthermore, ricin has been considered as a possible weapon for warfare and terrorist attacks. Although less potent than other biological toxic or infectious agents, it can be prepared more safely from easily available material with relatively simple equipment and procedures, thus without the support of a strong organization, and could be used for limited terrorist actions. Sources of other toxic RIPs are not as easily available as castor beans, but their toxins could be obtained by biotechnological techniques. Ricin and related toxins would be lethal in small amounts only if injected or inhaled but have a much lower toxicity by the oral route, and therefore could not be used to contaminate water supplies or large amounts of food. Aggressions to single persons could be performed by injecting a toxin, as occurred in London, when an expatriate Bulgarian journalist was killed with a microbullet containing ricin, as ascertained by the autopsy [205].

Serious concerns that ricin or similar toxins could be disseminated in the air as dust or aerosol in terrorist attacks prompted a number of studies. The consequences of ricin and abrin inhalation were investigated in rats. Damage was confined to the lung, where serious damage to

the lung epithelial lining was observed [206]. Interestingly, in an ultrastructural study by Assaad et al. [cited in ref. 206], damage to pneumocytes was seen as early as 15 min after inhalation of ricin, in the authors' opinion, too early to be due to inhibition of protein synthesis.

In our Department, the damage caused by ricin to rabbit eyes was studied, on the assumption that ricin disseminated in the air as powder or sprayed as an aerosol would reach the eyes [207]. The toxin caused severe inflammatory and even necrotic lesions, which could be greatly reduced by washing the eyes with a lactose solution, but only if was applied 1 min after the toxin, whereas after 5 min, there was very little protection. Washing with saline was ineffective, even if done 1 min after the toxin exposure. Together, these results indicate that (i) the toxin binds almost immediately to galactosyl-terminated receptors on the cell, from which it can be removed by lactose, but not by saline, and (ii) ricin enters cells rapidly, after which washing with lactose is almost totally ineffective.

The aim of several studies has been to detect and quantify ricin by methods which, ideally, should be sensitive enough, and also give rapid results if the diffusion of the toxin is suspected. Methods were developed based principally on immunoassays [208] and mass spectrometry analysis [209]. An immuno-PCR set up for the determination of viscumin [210] could also be employed. Furthermore, research on antibodies [211] and vaccines [212–215] has been performed to afford protection against ricin, with emphasis on the protection against the inhaled toxin [216].

Conclusions and perspectives

Studies on RIPs have considerably increased the knowledge in the field, and have changed our views about these proteins. The first consideration emerging from recent results is on the distribution of these proteins. Type 1 RIPs were already known to be widely diffused among plants, and now it appears that type 2 RIPs may be more frequent than was thought, possibly even toxic ones, since one of the latter, aralin, is present in a plant not known as toxic. Furthermore, it may be important to ascertain whether RIPs or similar proteins are produced by organisms other than plants, such as bacteria and possibly animals.

The mechanism underlying the toxicity and other properties of RIPs may also differ from what was originally thought. Some degree of RIP specificity is suggested by the observations that various RIPs bind to different ribosomal proteins and that the activity of some RIPs is stimulated by different tRNAs. Also, it is becoming apparent that the inhibition of protein synthesis consequent to damage to ribosomes cannot account for all the biological properties of RIPs, which are different from those of other inhibitors of protein synthesis. The action on DNA and other polynucleotide substrates remains to be clarified, and may have a role in some of the effects of RIPs, such as the antiviral activity, the pathogenesis of apoptosis, and the transforming activity. A significant role in the toxicity of RIPs is played by the activation of kinases, which induce the release of cytokines. These, in turn, may account for the inflammation caused by ricin and other RIPs and may also account for the alleged anticancer effect of some RIPs, e.g. viscumin. The activation of kinases seems to have a role also in the activity of trichosanthin and presumably other RIPs against HIV and possibly other viruses. On the basis of these recent studies, it may be worth investigating whether some effects of toxic RIPs may be alleviated by antagonists of cytokines and inhibitors of kinases.

The major challenge to investigators remains understanding the role of RIPs in nature. This must be important, to justify the conservation through evolution of proteins which are energetically expensive to synthesize. RIPs likely exert functions such as storage in seeds, defence against predators or parasites [reviewed in ref. 4], and may have a role in ageing or stress [36]. On the other hand, clearly none of these functions is exerted by RIPs in all producing organisms, which range from bacteria to plants and possibly animals.

Other questions concern the practical applications of RIPs. A possible utilization is in agriculture, to enhance plant resistance to viruses or other pests by transforming them with type 1 RIPs. This should be possible, and damage to transformed plants might be avoided with the use of appropriately mutated RIPs. Plants so modified should be safe for human health, certainly in the case of non-edible plants, and probably of plants which are eaten cooked, whereas the safety of those eaten raw will depend on the level and the possible allergenic properties of the expressed RIPs.

Another field still open is that of the immunotoxins and other conjugates. Although the hopes of their use in therapy raised by their availability proved to be excessive, they remain excellent experimental tools. With them, much valuable information has been obtained in neurological studies, and other applications will undoubtedly be found, for example for the removal of a given type of cell *in vitro*. As for therapy, it is possible that conjugates will be used under particular circumstances and with appropriate cautions.

A good deal of research has been done on RIPs and many results have been obtained which, however, are outnumbered by the unanswered questions they have raised. This is a common occurrence in science, and the investigator must humbly accept the replies given by nature, recalling Newton's words: 'I don't know what I may seem to the world, but, as to myself, I seem to have been only like a boy playing on the sea shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than

ordinary, whilst the great ocean of truth lay all undiscovered before me'.

Acknowledgements. The research performed in the authors' laboratory was supported by the University of Bologna, Funds for Selected Research Topics, the Ministry of Education, University and Research, the Ministry of Welfare, the Associazione Italiana per la Ricerca sul Cancro, and the Pallotti Legacy for Cancer Research.

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