Cytotoxicity and Toxicity to Animals and Humans of Ribosome-Inactivating Proteins

Maria Giulia Battelli*

Department of Experimental Pathology, Alma Mater Studiorum University of Bologna, Bologna, Italy

Abstract: The toxicity to cells and animals of type 1 and toxic and non-toxic type 2 Ribosome-Inactivating Proteins (RIP) is discussed in correlation with their catalytic activity, resulting in ribosome inactivation and apoptosis. The symptoms and histopathological lesions induced by RIP to animals and humans is also reviewed.

Keywords: Cytotoxicity, Toxicity to animals, Toxicity to humans, Type 1 ribosome-inactivating proteins, Toxic type 2 ribosome-inactivating proteins, Non-toxic type 2 ribosome-inactivating proteins.

The toxicity of castor and jequirity beans, as well as the abortifacient activity of some Cucurbitaceae, were known since ancient times, thus well before the identification of the proteins responsible for these biological effects. Most of the Ribosome-Inactivating Proteins (RIP) have been detected and purified after the cytotoxicity of ricin and abrin on experimental tumour cells was described [1]. On the basis both of protein structure and level of toxicity, RIP were initially classified in type 1 RIP, consisting of one peptide chain with a low toxicity, and two-chain type 2 RIP with a high toxicity (reviewed in [2]). Indeed, RIP have a highly variable toxicity to cells and animals (reviewed in [3]) and this variability may depend on differences either in the catalytic activity or in the interaction with cell or in the metabolism by the organism.

1. CYTOTOXICITY

The toxic effects of RIP were early attributed to the inhibition of protein synthesis [4, 5]. The identification of 60S ribosomal subunit as the target of ricin action [6] suggested the denomination of RIP for plant protein toxins, which cause the inhibition of protein synthesis by inactivating ribosomes [2]. After RIP's enzyme activity had been recognized [7,8], the rRNA *N*-glycosidase activity (EC 3.2.2.22) became the hallmark of RIP and is still used to identify these toxic proteins. More recently, the *N*-glycosidase activity of RIP was demonstrated to be able of deadenylating other substrates besides rRNA, in particular a common feature of all RIP consists in the depurination of DNA [9]. The observation that RIP also depurinates DNA and other nucleic acids (reviewed in [10]) has open new perspectives about the mechanism of cell killing by these toxins. This adenine polynucleotide glycosylase (APG) activity is not evenly distributed amongst different RIP, nor is correlated to the action on rRNA; however, it could result at least in part responsible for the differences in RIP cytotoxicity, when its role in cell killing would be clarified. The enzyme activity of different RIP may vary depending on structural differences both in the active site and in other domains of the activity-bearing protein chain, which determine substrate specificity, by influencing the interaction of each RIP with different subcellular targets.

1.1. Ribosome Inactivation

Most evidences about RIP's cytotoxicity were obtained by determining the incorporation of labelled amino acids by cell. Although HeLa cells are not very sensitive to RIP, this cell line was used in many cases to determine the inhibition of cell protein synthesis, allowing to compare the effect of different RIP (Table **1**). In this regard, it must be kept in mind that any comparison is somewhat arbitrary unless the experimental conditions are quite identical. In particular, the effect of RIP on cells may vary depending on: (i) the number of cells per well, (ii) the presence of serum in the medium, (iii) the time of exposure to RIP, and (iv) when the cells are pulsed with RIP for a short time, the length of the chase period. The measure of RIP action was obtained by calculating the IC_{50} (concentration of RIP required to inhibit protein synthesis by 50%). This value was assumed to be indicative of RIP toxicity since the kinetics of cell killing by abrin, ricin and modeccin showed that any reduction of cell protein synthesis was associate to a similar reduction in plating efficiency [11]. Indeed, this study ascertained that cells are killed by an all or none effect, thus the entry of a single molecule into the cytosol may be sufficient to kill a cell. The results of these kinetic studies are compatible with the catalytic nature of RIP, which is responsible for their toxic effects. The inhibition of protein synthesis precedes cell death and its determination is a very sensitive cytotoxicity test. As an example, the concentration of ricin or volkensin required for the inhibition of protein synthesis by 50% in microglial cultures was lower by about two orders of magnitude than that needed to obtain the same level of cell death after an equal exposure time [12].

The differences observed in the cytotoxicity of different RIP are higher than those described for the enzymatic activity, possibly because there are differences in their uptake and RIP have to reach the intracellular target to exert their toxic action. The entry process is mostly oriented by the toxin structure and greatly influences the toxicity level of each RIP. The difference in toxicity between type 1 and 2

*Address correspondence to this author at the Dipartimento di Patologia Sperimentale, Via San Giacomo 14, I-40126 Bologna, Italy; Tel: +39-051-2094729; Fax: +39-051-2094746; E-mail: mgbatt@alma.unibo.it

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Table 1. Range of Enzyme Activities and Toxicity to Cells and Animals of Type 1 and 2 Ribosome-Inactivating Proteins

	Type 1 RIP	Non-toxic type 2 RIP	Toxic type 2 RIP
RIP activity IC_{50}[1] (nM)	<0.01 - 4.0	2.7 - >100	43 - 88
Reduced lectins[2]	-	0.0003 - 8.2	0.1 - 3.5
APG activity[3] (pmol)	81 - 7,567	50 - 388	19 - 316
Cytotoxicity IC_{50}[4] (nM)	170 - >3,300	0.54 - >15,000	0.0003 - 1.7
Toxicity to mouse LD_{50}[5] (mg/kg body weight)	0.95 - 44	>1.6 - >40	0.0017 - 0.008

[1]Concentration giving 50% inhibition of protein synthesis in a cell-free system ([3] and Table 2). [2]Lectins were reduced with 1% 2-mercaptoethanol for 1 h at 37°C. [3]Adenine polynucleotide glycosylase activity on herring sperm DNA, expressed as adenine released ([9] and Table 2). [4]Concentration giving 50% inhibition of protein synthesis by HeLa cells after overnight exposure to RIP ([3] and Table 2). [5]Dose killing 50% of animals within one week after systemic administration of RIP ([3] and Table 2).

RIP was ascribed to the absence or presence of the B chain with lectinic properties (reviewed in [3]), which, ensuring the binding to the cell surface, facilitates the endocytosis process [13,14]. Comparing the cytotoxicity of various type 2 RIP for a cell line and, conversely, the different sensitivity of various cell lines to the same toxin, it appeared clear that the interaction between cells and RIP was more complicated than it was predictable on the basis of the molecular structure. Indeed, the presence of RIP-binding site on the cell surface does not guarantee that the toxin will be internalized and arrest protein synthesis [15]. Moreover, the cytotoxicity of RIP was not proportional to the number of membrane receptors. For instance, modeccin is as toxic to cells as ricin and abrin [16] and, like these toxins, it can bind to membrane molecules containing terminal galactose residues, although different from the abrin receptors and present in much smaller numbers. These findings indicate that cells possess different populations of binding sites with differences in the ability to facilitate the uptake of the toxins [17]. Indeed, it has been calculated that approximately only 5% of the total amount of ricin within the cells may reach the subcellular compartment that allows the translocation to cytosol [18].

The correlation between RIP structure and cytotoxicity had become even less linear when a new category of type 2 RIP emerged, which, in spite of the presence of the lectinic chain, have a low toxicity, similar to that of type 1 RIP (Table **1**). The lag time for the inhibition of protein synthesis by cells suggested that the toxins need to follow an intracellular path before reaching the ribosomal target [15]. Thus, the fate of RIP after internalization appears most relevant to their cytotoxicity (reviewed in [19]). For instance, the lower cytotoxicity of nigrin b compared with ricin has been at least in part explained by a higher degradation of nigrin b by cells, with a resulting lower concentration remaining inside the cells, and by the different intracellular pathways followed by the two lectins [20]. However, toxic type 2 RIP also has a highly variable toxicity, which could be accounted for mostly by differences in their intracellular fate.

Studies with different cell types showed a highly variable sensitivity toward RIP (reviewed in [3]). To understand the reasons for such a difference, mutant cells were obtained with various deficits in intracellular protein transport, being more resistant to RIP as compared to wild type cell lines [21,22]. Furthermore, substances altering the morphology and/or the physiology of subcellular compartments are able to modulate in a different way the sensitivity of cells to RIP (reviewed in [23]). The results of these studies suggest a close correlation between cytotoxicity and intracellular routing of RIP, which may vary within different cell types depending on: (i) the expression of different types of binding molecules on the cell surface, (ii) the sorting of ligands leading to different compartments, and (iii) the availability of various pathways for the transport of the toxin to the cytosolic target (reviewed in [24]).

Macrophages and trophoblasts have been found to be the most sensitive cells to RIP, possibly because of their ability to take up a wide variety of substances by different surface receptors. The high sensitivity of these cells confirms the relevance of the internalization manner in RIP cytotoxicity and suggests that this might be the reason for the immunosuppressive and abortifacient activities of these toxins (reviewed in [3, 25]).

1.2. Apoptosis

The first observation of apoptotic cell death caused by RIP has been reported in lymphatic tissues and small intestine of rats intoxicated with ricin and abrin [26-28]. *In vitro*, ricin, abrin and diphtheria toxin were found to induce programmed cell death in Vero and in MDCK cells [29] and in U937 human monoblastoid cell line [30]. Ricin-induced apoptosis was also described in cultures of T blasts [31], in peritoneal mouse macrophages [32], in a panel of tumor cells [33], and in bovine pulmonary endothelial cells [34]. DNA fragmentation, a characteristic feature of apoptosis, was also induced by ricin in various cell lines [35]. Moreover, endogenous proteases were activated in ricin-treated cells, and protease inhibitors prevented DNA fragmentation and cell death induced by treatment either with ricin, modeccin, Pseudomonas toxin, or diphtheria toxin [36].

Not only the holotoxin but also the isolated B chain of ricin, although devoid of protein synthesis-inhibiting activity, was able to induce apoptotic cell death, possibly by linking surface molecules which activate programmed cell death [37]. Furthermore, ricin A chain showed a cytotoxic activity to human umbilical vein endothelial cells that preceded the inhibition of protein synthesis [38] and appeared linked to a structural motif in the molecule, which

Table 2. Enzyme Activities and Toxicity to Cells and Animals of Type 2 Ribosome-Inactivating Proteins[1]

	RIP activity IC_{50}[2] (nM)		APG activity[3] (pmol)	Cytotoxicity IC_{50}[4] (nM)	Toxicity to mouse LD_{50}[5] (mg/kg)
	Native	Reduced			
Toxic type 2 RIP					
Abrin	88	0.5	168	0.0039 [16]	0.0028 [14]
Modeccin	45	2.3	19	0.0028 [16]	0.0053 [52]
Ricin	84	0.1	316	0.0007 [53]	0.0080 [13]
Viscumin	43	3.5	112	1.7 [16]	0.0024 [54]
Volkensin	84	0.4	74	0.0003 [55]	0.0017 [55]
Non-toxic type 2 RIP					
Cinnamomin	30.5	7.4	95	18.8[6] [56]	-
Ebulin L1	>100	0.15	325	62 [53]	>2 [57]
IRA b	2.9	3.6	367	108[6] [58]	-
IRA r	5.8	4.7	388	82[6] [58]	-
Nigrin b	>100	5.2 [57]	242	53.6 [20]	12 [20]
Nigrin b (basic)	-	0.0003 [59]	-	>15,000 [59]	>40 [59]
Nigrin f	-	0.03 [60]	-	2.9 [53]	>1.6 [60]
RCA 120	>100	0.05	155	0.542 [61]	1.4 [62]
Sieboldin b	-	0.015 [63]	-	11.8 [63]	>1.6 [63]
SNA I	2.7	1.65	50	>400 [64]	-
SNLRP	6.0 [64]	5.7 [64]	192	>1,500 [64]	-
SSA	-	8.2 [65]	-	458 [63]	>4.2 [63]

[1]Only type 2 RIP with comparable cytotoxicity and toxicity to animals were included.

[2]Concentration giving 50% inhibition of protein synthesis by a cell-free system was determined as described [49]. Lectins were reduced with 1% 2-mercaptoethanol for 1 h at 37°C (Prof. Fiorenzo Stirpe, personal communication).

[3]Adenine polynucleotide glycosylase activity on herring sperm DNA, expressed as adenine released, was determined as described [9] (Prof. Luigi Barbieri, personal communication).

[4]Concentration giving 50% inhibition of protein synthesis by HeLa cells after overnight exposure to RIP.

[5]Dose per body weight killing 50% of animals within one week after systemic administration of RIP.

[6]Cytotoxicity was determined as inhibition of cell proliferation on human cell lines.

is far apart from the active site of ricin [39]. Thus, apoptosis appeared independent of the inhibition of protein synthesis by RIP, since this and the injury of endothelial cells were not mediated by the same portion of RIP molecule. This short amino acid sequence is shared by other proteins, including cytokines and adhesion molecules, which could be responsible for the vascular leak syndrome and may be involved in its occurrence in patients treated with RIP-containing immunotoxins. This sequence could be responsible for caspase-3-mediated apoptotic action of ricin A chain or ricin A chain-containing immunotoxin on endothelial cells, by competing for cells binding to adhesion molecules [39]. Ricin may exert its cytotoxicity either by activating the caspases pathway leading to DNA fragmentation or by NAD^+ and ATP depletion, which can be prevented by the inhibition of poly(ADP-ribose) polymerase [40]. Thus, multiple apoptotic signaling pathways may be triggered by ricin-treatment.

Mistletoe lectins were recognized to induce apoptosis in a variety of tumor cell lines [41] and in cultured human lymphocytes, as measured by the appearance of a hypodiploid DNA peak using flow cytometry [42]. RIP from *Viscum album* L. and volkensin also induced the expression of mitochondrial membrane protein Apo2.7 in lymphocytes from healthy subjects [43]. Consistently, the activation by mistletoe lectin I of caspases in the mitochondria-controlled apoptotic pathway was reported, whereas no receptor-triggered apoptosis was detected in lymphoid cell lines [44].

Type 1 RIP also induced apoptotic DNA alterations, observed with a PAP-containing immunotoxin [45] and agrostin [46] on leukemic cells. Exposure to saporin induced apoptosis in different cellular models, such as human peripheral blood B lymphocytes and neutrophils, in the Daudi B-cell line, and in the hemopoietic cell lines HL-60 and TF-1 [47]. Momordin, PAP-S and saporin were shown

to induce apoptotic death of CD30+ L540 cell line [48]. The same RIP were conjugated to a monoclonal antibody recognising the CD30 antigen of human lymphocytes. The resulting immunotoxins caused apoptosis in 50% of cells at a concentration lower by three orders of magnitude as compared with that required in the case of free RIP. These findings may be relevant to the therapeutic use of RIP-containing immunotoxins, which could be devoid of more extensive tissue damaging effects as would be the case if they induced only necrosis of target cells [47].

Immunotoxins containing saporin and bouganin inhibited cell protein synthesis, induced apoptosis and blocked the clonogenic growth of target cells, although with a different kinetics, slower in the case of conjugated bouganin [49]. The lack of a complete correspondence between the three biological effects may reflect the presence of different mechanisms of cell killing acting through the deadenylation of rRNA or DNA or other nucleic acid target. DNA depurination by RIP has been suggested to have a role in the pathogenesis of lesions induced by RIP, and particularly in the DNA fragmentation typical of apoptosis [9]. Ricin was shown to damage nuclear DNA in whole cells by means that are not secondary to ribosome inactivation or apoptosis, confirming a mechanism of apoptosis centred on the enzymatically induced DNA lesion [50]. It was also suggested that a failure of the suicide program in cells intoxicated with RIP might lead to mutagenic effects. Indeed, a new biologic activity of RIP has recently been described, i.e. the ability to induce transformation of fibroblasts, possibly as a consequence of damage both to DNA and poly(ADP-ribosyl)ated poly(ADP-ribose) polymerase; the latter being involved in the DNA repair system [51].

2. TOXICITY TO ANIMALS AND HUMANS

The toxicity of RIP to animals is highly variable, although type 1 RIP and the A-chains of type 2 RIP share the same catalytic activity. Wide differences in the inhibition of cell-free protein synthesis and DNA depurination are described amongst type 1 RIP, as well as inside toxic and non-toxic type 2 RIP (Table **1**). However, the level of these enzyme activities is not predictive of RIP toxicity either to cells or to animals. A correlation can be observed between cytotoxicity and toxicity to animals of RIP, *i.e.* the difference in toxicity to cells between different types of RIP reflects a parallel difference in toxicity to animals. Type 1 and non-toxic type 2 RIP have a similar cytotoxicity in a μmolar range, which is three orders of magnitude lower than that of toxic type 2 RIP. Likewise, the LD_{50} of toxic type 2 RIP is in the same range (μg/kg body weight), while that of type 1 and non-toxic type 2 RIP is higher by at least three orders of magnitude. To allow a better comparison, the information about individual type 2 RIP has been collected in (Table **2**), whenever results on cytotoxicity and toxicity to animals were available.

The low toxicity to animals of type 1 RIP is mainly due to the absence of the lectinic chain, resulting in a low grade of penetration into cells. In spite of the similarity in the structure and the apparently identical mechanism of action, the toxicity and the lesions caused by various type 2 RIP are different. The lack of toxicity of the non-toxic type 2 RIP could be ascribed to characteristics of B chain, which may influence the subcellular distribution and thus the fate of the A chain after internalization. Recently, ricin B-chain has been described to have a lipase activity, essential for A-chain translocation into cytosol and cytotoxicity [52]. Mutation and structural studies suggest that the presence or the absence of such activity could justify the different toxicity of toxic and non-toxic type 2 RIP. The different effects and lesions observed *in vivo* after the administration of various toxic type 2 RIP could be also due to diversity of their B chains, which may affect (i) the penetration into the cell, (ii) the intracellular pathway and fate, and (iii) the distribution among different cell types, and consequently among different organs.

2.1. Toxicity of Type 1 Ribosome-Inactivating Proteins

The blood clearance and organ distribution of several type 1 RIP (bryodin, gelonin, momordin, PAP-S, saporin-S6, trichokirin and momorcochin-S) in mice, as well as the lesions caused by lethal doses of them, were described in comparative studies. RIP showed a blood half-life of 4-8 min, and were concentrated mainly in the kidney [67]. The organs constantly involved were liver, kidney and spleen, and the lesions were essentially cell necrosis, sometimes accompanied by fatty change [68]. Non-lethal doses of various type 1 RIP did not caused significant permanent lesions in mice after 14 days of treatment [3].

The *in vivo* uptake of saporin-S6 by rat liver was mostly performed by non-parenchymal cells, which accumulated 25 times more RIP than parenchymal cells [69]. Saporin-S6, at doses toxic to rat, induced an increase of xanthine oxidoreductase in serum, possibly because the enzyme was released from damaged liver cells [70]. Acute renal failure in rats has been reported to be caused by trichosanthin injection, which induced proximal tubule lesions, including both necrotic and apoptotic cell death [71]. Toxicity to kidney could be attributed to tubular reabsorption of trichosanthin.

No fatal poisoning in humans by type 1 RIP was reported when trichosanthin was given to induce abortion [72] or as an antiviral drug in AIDS patients [73-75]. Side effects of RIP-induced pregnancy interruption were: fever, headache and soreness of joints, which disappeared after 48 hours. AIDS patients treated with trichosanthin developed an influenza-like syndrome with fever, rashes, myalgias, arthralgias, as well as signs of liver toxicity and neurological adverse reactions. The latter included headache and a transient dementia sometime progressing to reversible coma, and in one case, to acute disseminated encephalomyelitis [75]. Two cases of multifocal neurological deficits have also been reported after trichosanthin treatment in HIV-infected patients [76].

2.2. Toxicity of Type 2 Ribosome-Inactivating Proteins

Toxic type 2 RIP are among the most potent known toxins, though, the lethal doses of toxic type 2 RIP may vary greatly among different species, as reported for abrin and ricin (reviewed in [77]), modeccin [78] and volkensin [55]. The precocity and severity of the lesions induced by

RIP depend on the dose. However, rats given high doses of these toxins had died within 6-10 h, before lesions accounting for death were detectable in parenchymal organs. This lag time is always observed, even with supralethal doses of abrin and ricin [79], modeccin [78,80], viscumin [54] and volkensin [55].

The toxicity of the seeds of *Abrus precatorius* L. and of *Ricinus communis* L. to humans and animals has been historically utilized in folk medicine and for criminal purposes [81]. Symptoms and alterations observed in about 700 people poisoned with castor beans were reported [82]. The most frequent signs concerned the gastrointestinal tract (nausea, thirst, bloody diarrhoea and liver necrosis), the nervous system (headache, somnolence, loss of consciousness, convulsions, optic nerve lesion and mydriasis) and the kidney (nephritis and proteinuria), although systemic modification, like shock and changes in the ECG were also described. Pathology included bleeding in the serous membranes, multiple ulcera and hemorrhages in the stomach and intestine, degenerative changes in the heart, liver and kidney, and damage in the spleen and lymph nodes.

The symptoms, gross lesions, and microscopic alterations caused by abrin and ricin in animals have been described since the end of 19th century. Lethal doses of abrin, given by subcutaneous injection, induced a progressive drowsiness, lowering of the body temperature, anorexia, tube casts in the urine and blood in the faeces in rabbits [83]. Orally administered sublethal doses caused diarrhoea, loss of weight and eventually the tolerance. Gross pathology mainly consists in hyperemia in parenchymal organs, with small hemorrhagic effusion, enlargement of lymphoid tissues, damages to the digestive mucosa, and the occurrence of slightly hematic fluid in pericardium and lung bases.The histological examination showed necrosis of intestinal epithelium, and hydropic and fatty degeneration of cardiac cells, which could impair heart function. The toxic effects of abrin and ricin in rabbit and guinea pig were also reported, confirming many of the above observations with the addition of severe convulsions and hemorrhagic ascites [84]. The occurrence of necrosis was described in macrophagic and endothelial cells of lymphnodes and spleen, as well as in epithelial cells of intestine, liver and kidney, and in muscular cardiac cells. Also, damages to neuronal body and dendrites were observed in the brain of animals poisoned with ricin.

The highest concentration in mouse tissues of injected abrin or ricin was found in spleen, followed by kidneys, heart, liver and thymus. However, the relative concentration in liver was considerably higher for ricin than for abrin [79]. Weakness, anorexia, weight loss, and moderate fever were the main findings in dogs given lethal doses of abrin or ricin [85]. Light and electron microscopic examination only showed evidence of increased phagocytic activity of reticuloendothelial cells in dog liver and spleen.

Poisoning of mouse with lethal doses of abrin caused large areas of ulceration in the intestine and marked congestion in various organs, including liver, kidney, heart, lung, spleen, cerebellum and lymphnodes [86]. Abrin brought about only severe necrosis of the acinar pancreatic cells and impairment of protein synthesis in the pancreas of poisoned rats [87]. Moreover, abrin induced apoptotic changes in the lymphoid tissues and the intestine of the rat [26]. Apoptotic changes were also described in the lesions induced by abrin and ricin in the lymphoid tissues and intestinal epithelial cells of rats [26, 28], as well as by ricin in the thymus and spleen of mice [88].

The toxicity of ricin to rat organs was investigated [89]. The earliest changes induced by ricin administration were observed in sinusoidal cells, which had been progressively damaged until became necrotic. Only after the development of these lesions, hepatocytes appeared to be damaged, suggesting that hepatocytes necrosis is a consequence of the destruction of sinusoidal lining and of the following thrombosis. A severe necrosis of the red pulp of the spleen rich in reticuloendothelial cells, was also observed. Indeed, a marked and precocious inhibition of protein synthesis was observed in the spleen of rats poisoned with ricin.

The toxic effect of ricin A chain administered at non-lethal doses was studied in rats and monkeys [90]. Hematological alterations included decrease in erythrocytes, polymorphonuclear neutrophils and platelet counts, lymphocytosis, and elevation of serum markers of liver damage. Necropsies revealed centrilobular necrosis of the liver, some necrosis of the proximal tubules of the kidneys and lesions of the serous acini of salivary glands and pancreas.

The distribution of [^{125}I]-ricin was studied in mice following aerosol inhalation exposure [91]. Radioactivity mostly accumulated in lung and in the gastrointestinal tract. Only small amounts of ricin delivered to the gastrointestinal tract were absorbed into the circulation and recovered in other organs. Acute inhaled lethal ricin intoxication induced histopathological lesions restricted to the lung, which were similar in mouse [92, 93], rat [94, 95] and rhesus monkeys [96]. Animals developed diffuse pneumonia resulting in airway epithelial necrosis, interstitial and intra-alveolar oedema and accumulation of inflammatory cells involving all lung lobes, and died 48-96 hr after aerosol exposure to ricin because of the resulting hypoxia. Electron microscopy showed nuclear apoptotic changes in alveolar macrophages, necrosis of capillary endothelium and type I epithelial cell, and evidence of microvascular microthrombosis [95]. In a comparative study the effects of inhaled abrin were similar to those of ricin, although the appearance of apoptosis was far more marked following inhalation of ricin than of abrin. At a difference with ricin-intoxicated rats, abrin-exposed animals had marked intra-alveolar and interstitial hemorrhage [94].

The ingestion of the root of *Adenia digitata* Burtt-Davy was known by the South African natives to cause severe disease and even death. The progress of the intoxication depends on: (i) the variable presence of a cyanogenetic component, which causes short-term lethality, and (ii) the dose and way of administration, which modulate the effects of modeccin, responsible for damages and death at later time. The occurrence of accidental human poisoning, which caused an acute hemorrhagic gastro-enteritis and death, stimulated the study of *Adenia digitata* toxicity. The effects of an aqueous extract of dried root were investigated in various animals, including dog, guinea pig, rabbit, sheep and rat [78]. The typical lesions caused by oral

administration were acute, sometimes hemorrhagic, gastro-enteritis, whereas subcutaneous injection mainly induced liver and kidney congestion and lung oedema. A slight biliuria and albuminuria were reported in rabbit and reflected the alteration of liver and kidney.

Like *Adenia digitata*, *Adenia volkensii* Harms have a poisonous tuberous rootstock, containing a cyanogenetic glycoside and a toxic type 2 RIP, volkensin. The high toxicity of the plant to animals and humans was known by the indigenous population of Kenya. The pathological changes induced by feeding with an aqueous extract of *Adenia volkensii* were described in rats and sheep [97]. Congestion and hemorrhage were observed in gastrointestinal tract, lungs, liver and kidney, with frequent hyaline casts and blood in the urine. Meningeal congestion and oedema often occur, as well as subepicardial and myocardial hemorrhage.

In rats poisoned with modeccin [55] and volkensin [80] given intraperitoneally, the most severe injuries were observed in the liver, in which they caused serious, up to necrotic, lesions. These alterations were similar, although not identical, to those brought about by ricin, which appeared to affect hepatocytes as a consequence of the destruction of non-parenchymal cells. Changes in the rough endoplasmic reticulum and swelling of mitochondria were well marked in the hepatocytes as early as 6 hours after poisoning with modeccin [98]. In parallel with the occurrence of morphological lesions, protein synthesis was impaired in the liver of rats poisoned with modeccin, and this effect was accompanied by damage of the 60 S ribosomal subunit. Thus modeccin, and presumably the other toxins damage ribosomes *in vivo* in the same way as they do *in vitro*.

The poisonous effects of *Viscum album* L. have been noticed since pagan times, when it was considered a holy plant and reported in Nordic mythology. Extracts from mistletoe have been used to prepare herbal remedies against a variety of diseases including cancer. A potential use of these preparations to modulate the immune system has been suggested, in view of their ability of stimulating the cellular parameters of natural immunity, by increasing the secretion of proinflammatory cytokines (reviewed in [99]). However, the experimental results are still waiting for a clinical confirmation based on direct anticancer action or improvement in time to tumor progression or overall survival in cancer patients (reviewed in [100]). In spite of the lack of a demonstrated efficacy, herbal remedies containing mistletoe extracts have been used, mostly in cancer therapy, although suspected to cause damage to liver by inducing hepatitis [101]. A *Viscum album* extract, administered subcutaneously as an antiviral drug, induced no severe side effects in HIV-positive patients and healthy participants to a dose-escalating phase I/II study [102]. However, flu-like symptoms, fever and eosinophilia occurred, as well as an increase of serum levels of urea nitrogen and creatinine.

Lesions observed in rats poisoned with lethal doses of viscumin include ascites, congested intestine and hemorrhages in the pancreas, which are similar to those caused by ricin [103]. However, as in the case of abrin, no lesions were detected, which could justify the death of poisoned rats.

3. TOXICITY-RELATED BIOLOGICAL ACTIVITIES

RIP have various biological activities, which are in some way related to their toxicity, including (i) inducing abortion, (ii) modulating the immune response, and (iii) be retrogradely transported along the axon to neurones.

3.1. Abortifacient Activity

Extracts of *Trichosanthes kirilowii* and of other Cucurbitaceae have been utilized since ancient times in China for their abortifacient action [104] and are still an object of clinical studies not only for termination of early and midtrimester gestation, but also in the therapy of ectopic pregnancy, hydatidiform mole, invasive mole and choriocarcinoma (reviewed in [25,105]). This biological activity is due to proteins like trichosanthin, alpha-momorcharin and beta-momorcharin, which were proven to be RIP [106], and, conversely, other RIP showed abortifacient activity in pregnant mice. The induction of abortion seemed correlated with a selective toxicity toward the trophoblastic tissue, which has been ascribed to RIP [107, 108]. A summary of 402 cases of abortion induced by trichosanthin was described [109]. The mechanism of action lies in the selective damage of placental villi, which causes the coagulation of blood sinus [110]. Indeed, the main pathological findings are necrosis of the syncytiotrophoblast and thrombosis of the intervillous spaces [111]. As a consequence of the circulation hindrance, tissue necrosis over large areas follows inducing the impairment of functional activities and the lowering of steroid hormones, which are essential for pregnancy to go on. Consistently, luffaculin, luffin-a, luffin-b and momorcochin administered i.p. to mice elicited a reduction in the circulating titre of estradiol-17 beta [112].

3.2. Immunogenic and Immunomodulating Activity

All RIPs are strongly immunogenic: the first report dating to experiments on the lack of cross-reactivity between abrin and ricin [113]. Rabbits immunized with formaldehyde-treated ricin produce polyclonal antibodies recognising both A and B chains of the toxin [114]. Anti-ricin sera neutralised effectively the ability of ricin to inhibit protein synthesis in HeLa cells and in a cell-free system. Antibody formation against abrin and ricin has been observed in mice and in cancer patients repeatedly treated with therapeutic doses of the RIP [115]. Mice developing anti-ricin immune response were protected from lethal doses of the toxin. Moreover, the administration of rabbit antibodies against ricin or its constituent polypeptide chains protected mice from ricin intoxication [116]. Humoral immune response has also been induced in rabbits against various type 1 RIP, namely dianthin 32, lychnin and saporin-S6 from Caryophyllace, bryodin-R, colocin 1, momorcochin-S, momordin, and trichokirin from Cucurbitaceae and PAP-R from Phytolaccaceae [117]. Sera of immunized rabbits strongly cross-reacted with RIP from plants belonging to the same family, whereas no cross-reactivity between RIP from taxonomically unrelated plants was observed.

The immunogenicity of a RIP containing-immunotoxin was observed in rats after multiple-dose administration

[118]. The treatment induced transient systemic effects such as peripheral oedema, leukocytosis, alterations of liver function tests and histopathological changes. These included cytoplasmic vacuolization of hepatocytes, focal myocardial and skeletal muscle degeneration, and renal deposits of proteinaceous casts. The development of host antibodies against RIP was also observed in patients treated with RIP-containing immunotoxins and might limit their therapeutic use [119]. Moreover, some of the patients involved in those clinical trials developed immune response causing adverse allergic reactions.

The allergenic action of castor bean was already known [120], mostly because of the occurrence of occupational allergy to castor bean in industrial workers [121]. Ricin was identified as the component of castor bean extract which induced the IgE response [122]. Moreover, ricin enhanced the responses to other antigens administered at the same time, acting as an IgE-specific adjuvant. A similar adjuvant activity was reported for *Viscum album* type 2 RIP, which enhanced in mice immune responses to mucosal co-administered antigens [123]. Mistletoe lectins induced high levels of mucosal IgA and systemic IgG with a high IgG_1:IgG_{2a} ratio, which is compatible with the selective induction of Th2-type immune responses. Also type 1 RIP were shown to be allergenic, since trichosanthin, alpha-momorcharin and beta-momorcharin induced IgE antibody response in mice [124]. Furthermore, trichosanthin induced IL-4 and inhibited IFN-gamma gene expression in mice and had an adjuvant action by bringing out the IgE response to ovalbumin [125]. Consistently, trichosanthin-treated peritoneal macrophages modified the response to LPS by upregulating the expression of IL-10 and MCP-1 and decreasing the production of IL-12 and TNF: effects that are likely to facilitate the induction of Th2 and IgE response [126]. Allergic reactions were also reported in AIDS patients treated with repeated doses of trichosanthin [74].

The pyrogenic effect of ricin injected to cat, guinea pig, rabbit and rat was described [127] and the production of pyrogenic cytokines was reported as a consequence of ricin treatment of rabbit white blood cells. In a further study, ricin given intraperitoneally in sublethal doses caused fever in a dose-dependent manner in rabbit, cat, guinea pig, rat and monkey [128]. Experiments with human peripheral blood mononuclear cells (PBMC) showed that a lectin from *Viscum album* [129] and ricin [130] induced the release of TNF-alpha. The enhanced expression of cytokines appeared involved in the toxicity of ricin, since anti-TNF-alpha antibody significantly reduced (i) its toxicity to a macrophagic cell line [131], and (ii) the urinary excretion of malondialdehyde, formaldehyde, and acetone induced by ricin in mice, as well as hepatic lipid peroxidation and DNA single-strand breaks [132]. Consistently, incubation of PBMC with ricin activated their cytolytic activity to K562 cell line as well as the secretion of IL-1 beta, IL-2 and TNF-alfa [133]. When injected per i.p. in mice, a partially purified protein factor from the bitter melon elicited the activation of peritoneal exudate cells, which were cytotoxic to tumor cell lines [134].

At low concentrations ricin potentiated the effect of lipopolysaccharide to induce IL-1, whereas higher concentrations of the toxin inhibited the production of IL-1 and the mitogen-driven proliferation of PBMC cultures [135]. This finding is in agreement with the observations about the high sensitivity to RIP of macrophagic cells [136-139], including microglial cells [12], and supports the hypothesis of an immunosuppressive activity of RIP (reviewed in [3]). Indeed, injection to mice of non-toxic doses of various RIP, namely momordin I and PAP-S [140], trichosanthin [141], alpha- and beta-momorcharin [142], PAP-1, PAP-S and ricin A-chain [143], had a significant immunosuppressive effect both on the humoral antibody formation and a variety of cell-mediate responses.

3.3. Axonal Retrograde Transport and Neurotoxicity

The lack of lethal lesions caused by abrin and viscumin suggests that these RIP may bring about some kind of undetected damage, for instance in the nervous system. This notion is indirectly supported by the observations on the extreme toxicity of ricin injected intraventricularly, which impaired incorporation of amino acids *in vivo* into brain total protein and into brain ribosomes, as well as protein synthesis *in vitro* by microsomes isolated from the brain of poisoned rats [144]. Moreover, the retrograde transport of ricin and the toxic effects in the autonomic nervous system were reported [145]. Modeccin and ricin [146] as well as volkensin [147] and RCA [148] injected into peripheral nerves could be retrogradely transported along the axon to the neurones, which were killed. Furthermore, modeccin and volkensin but not abrin were effective suicide transport agents in rat CNS [135]. Axonally transported toxins can be used to make selective lesions of the nervous system, which may be proven useful at least as an experimental tool, and perhaps as a molecular neurosurgery. Remarkably, ricin injected outside nerves could diffuse into tissue and be taken up by nervous terminations, and the suicide transport was not arrested by anti-ricin antibody, which had protected against systemic toxicity [149]. The abundant literature on this field has been discussed in some reviews, even recently [150-154].

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