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BIOLOGICAL AGENTS
Ricin – A research review [1]

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ABSTRACT
Ricin is a toxin of plant origin which causes fatal permeability-type (non-cardiogenic) pulmonary oedema on inhalation of the aerosol. Ricin is composed of two chains, linked by a disulfide bond. The B-chain acts to bind the toxin to cell membranes and assist in internalization of the A-chain, which inhibits protein synthesis by enzymatic action on ribonucleic acid. There is no known effective therapy for ricin intoxication. However, immunization is feasible.

INTRODUCTION
Ricin is a toxic protein and lectin extracted from the endosperm cells of the seeds of the castor oil plant, Ricinus communis. This plant is cultivated commercially for its oil but is also widespread as a weed and as an ornamental plant. It flourishes in most subtropical and some temperate climates.' Ricin was considered a candidate warfare agent during World Wars 1 and 11 and after World War II. Under the 1993 Chemical Weapons Convention, ricin is classified as a Schedule I chemical, defined as one which has been developed, produced, stockpiled or used as a chemical weapon specifically to cause death or other harm through its toxic properties. Ricin consists of two haemagglutinins (RCL 1 and 11) and two toxins (RCL III or D and IV). The agglutinins are tetramers (MW 130,000) and the toxins are dimers (MW 66,000).[2] Ricin shares a similar general structure and function with several other dimeric toxins. Each toxin is composed of two small (approximately 32 and 34 kD) protein chains, denoted A and B, joined by a disulfide bond.

TOXICITY
Ricin is an extremely toxic substance. A few picograms of the toxin per millilitre will kill most cells in culture within 24h[3]. One internalised molecule of ricin is sufficient to kill a cell, [1.1.6] while it has been estimated that the minimum amount of toxin to kill HeLa cells in culture is do molecules per cell.' Ricin inhibits the growth of various murine tumours and of several human tumour xenografts in athymic mice.[8] It appears to be more toxic to certain malignant cells than to normal cells[4]. Ricin is also immunogenic, and the dust generated from the extraction of castor oil from castor bean seeds is a potent allergen[6,9]. Ricin has no effect on the growth of bacteria.[3]

Table 1 lists the lethal doses of ricin in different species. Ricin is toxic parenterally, orally and by inhalation, and the parenteral and inhalation toxicities are similar. On a weight basis, the guinea pig is more sensitive to ricin than the mouse, while the horse is even more sensitive[4]. Different strains of mice show different sensitivities to ricin, DBA mice being the most sensitive. These inter-strain differences in sensitivity correlated with strain differences in the ricin concentration reached in important tissues.[8]

When introduced intravenously or intramuscularly, the primary targets of ricin are the liver (where up to 50 percent of injected ricin is retained), kidney and spleen.[10.11.13] Most of the toxin is excreted within 24h in the urine[13]. Lesions provoked by ricin have been studied extensively and all reports mention the necrosing effects on blood vessels and organ parenchyma, mainly in liver and lymphoid tissues. Large

doses cause haemorrhages in the viscera and serous cavities[7]. By contrast with the parenteral routes, exposure via the inhalation route results in the primary lesions being in the respiratory system, and death results from oedema and hypoxia. Assaad *et al* concluded that ricin induced-pulmonary toxicity may be divided into three phases: latent period, redistribution of lung water without an increase in extravascular lung water and pulmonary oedema.[14]

SPECIES	ROUTE	LETHAL DOSE	REFERENCE
Mouse CD-1	ip	LD50 7.5 mg/kg LD50 16mg/kg (A-chain) LD50 8mg/kg (B-chain)	Richer *et al*[10]
Mouse	ip	LD50 100 ng	Olsnes *et al*[7]
Mouse	iv	55-65 ng 2.7 mg/kg 65 ng	Foodstad *et al*[11] Hewetson et al[6] Olsnes[3]
Mouse	inh	LD99 10mg/ 1 air/10 min	Hewetson *et al*[6]
Human	oral	30mg, 0.03-1 mg/kg	Klain and jaeger[12]

TABLE 1. Lethal Doses of Ricin
Notes:

- *inh: inhalation*
- *intravenous*
- *ip: intraperitoneal*
- *LD50 or LCt50 – dose required to kill 50% of the population*
- *LD99 – dose required to kill 99% of the population*

The whole ricin toxin is 2,000 and 1,000 times more toxic than the A-chain and B-chain respectively. However, all the elements of the lesions induced by ricin can be found qualitatively in the effects of each subunit injected independently in mice. [10] if the separated A – and B-chains are combined again, the reconstituted protein is almost as toxic as the native protein, demonstrating that the peptide chains do not lose their activity during separation[7,15]. Further, Lewis and Youle showed that the A – and B-chains associate reversibly, without disulfide linkage, and that the association increases with increasing concentration in accordance with thermodynamic principles[15]. Using inhibition of protein synthesis in cell cultures as a measure of toxicity, they found that disulfide-reduced, but reversibly-associated ricin, with most of the ricin in the associated state, was as toxic to the cells as native ricin. They reached the conclusion that the disulfide bond linking the A and B subunits appears to play no role in toxicity in this system other than to hold the two subunits together at low concentrations. It should be noted however that there is no direct correlation between toxicity in animals and in cultured cells. [4] In intact cells and living animals, only molecular species with the disulfide bridge intact have toxic effects. Different cell lines differ in their sensitivity to ricin. Ricin also inhibits protein synthesis in cell-free systems (eg from rabbit reticulocytes), where the reduced toxin is active but not the native toxin, in contrast to the situation in vivo.

For parenteral administration, there is a latent period of 18 to 24h during which no signs of poisoning are discernible, even for high doses of toxin. Thereafter, animals show general malaise, piloerection and marked lethargy and anorexia. Other symptoms are diarrhoea, weight loss and moderate fever.[4] No animals died less than 8 to 10h after intraperitoneal administration of ricin in the study of Olsnes et al[7]. Survival time is dose-related, and the dose-response curve is steep[4].

CLINICAL MANIFESTATION

For poisoning of humans by ricin, the most famous case is that of the Bulgarian journalist Georgi Markov, who was killed by a pellet containing about 500 mg of ricin, which was implanted in his thigh via the tip of an umbrella. Five hours later he began to feel weak, and later developed a fever which continued the next day. Before death, there was a fall in temperature and in the blood pressure.[1-4, 12]

In most cases, however, ricin poisoning in humans is due to the ingestion of castor seeds. If they are well chewed, 2 or 3 seeds may be fatal to a child and 8 seeds to an adult. There is a latent period of 3 to 20h before the manifestation of toxicity. The symptoms include internal haemorrhage and gastrointestinal irritation, nausea, violent vomiting, abdominal pain, severe diarrhoea, dilatation of the pupils, and shivering. Convulsions occur in severe poisoning.[12.1] The quantity of ricin available for absorption depends on the degree of mastication of the castor seeds. It resists the action of proteolytic enzymes in the intestinal tract and presumably is absorbed without being hydrolysed. Thus, it is difficult to estimate the lethal oral dose of ricin for humans.[12]

MECHANISM OF ACTION AND THERAPY

The main function of the B-chain of ricin is believed to be binding of the whole toxin to target cell membranes (eukaryotic cells) by lectin-type recognition of b-D-galactose contained in glycoprotein or glycolipid sequences, and the internalisation of the toxin by endocytosis. Not all eukaryotic cells are sensitive, and no prokaryotes are known to be sensitive[17]. The A-chain has highly specific N-glycosidase activity and hydrolyses the N-glycosidic bond between the base (adenine) and the ribose at position A4324 in 285 ribosomal RNA (of a 605 ribosomal subunit); this renders the surrounding phosphodiester bonds highly susceptible to hydrolysis. The end result is therefore similar to that from the action of another cytotoxic protein, a-sarcin, which catalyses hydrolysis of the phosphodiester bond between A4324 and G4325. The hydrolysis prevents binding of an elongation factor and thereby inhibits protein synthesis.[17.18.19.20]

The sequence of events is, therefore: [21]

1. binding of the toxin to cell-surface receptors
2. receptor-mediated endocytosis and intracellular transport through the vesicular system
3. translocation of the enzymatically active component of the toxin (A-chain) across the vesicle membrane to the cytosol, following reduction of the inter-subunit disulphide bond [17]
4. enzymatic inactivation of the intracellular target.

Prophylaxis or therapy of ricin intoxication could therefore be directed at one or more of these four stages. A fifth possibility is inactivation of ricin in vivo by chemical means or immunologically. This would need to be prophylactically, or immediately after intoxication, although confirmation of intoxication will be difficult in the absence of immediate symptoms- compare nerve agent poisoning. Apart from the latent period before the effects of ricin are manifest, the clinical features of poisoning themselves give no clue as to the diagnosis.

A brief summary of research on the above stages in intoxication is as follows.

BINDING OF THE TOXIN TO CELL- SURFACE RECEPTORS

Ricin B-chain contains two galactose-binding sites.[20] Moreover, both A- and B-chains are glycosylated and expose terminal mannosyl residues, which can mediate binding of ricin to endocytic mannose receptors on cells that carry those, i.e. macrophages and liver endothelial cells.[22] Jang and Kim reported that the haemagglutination activity of ricin was inhibited by lactose, as well as by galactose and other sugars[23] Fodstad *el al* reported that lactose inhibited binding of the B-chain of ricin, and mice were partially protected from ricin toxicity by injection of lactose with the ricin." By contrast, Wannemacher et al reported that neither lactose nor the more stable synthetic disaccharide analogue lactulose was effective in protecting mice against ricin, although these compounds did reduce ricin-induced cytotoxicity in vitro.[24]

Wales *et al* analysed the galactose-binding site by X-ray and mutational analysis and identified key residues which hydrogen-bond to the sugar, and a conserved tripeptide Asp-Val-Arg in the binding site.[25] Newton *et al* studied the role of galactose binding sites of ricin A-chain in ricin toxicity by looking at a series of ricin point mutants.[26] The cytotoxicity was evaluated when cell entry was mediated either by galactose-containing receptors or through the alternate mannose receptor of macrophages. Even for mannose receptor-mediated toxicity of ricin, at least one galactose binding site remains necessary for cytotoxicity and two galactose binding sites further increase potency. These results are consistent with the model that the ricin B-chain galactose binding activity plays a role not only in cell surface binding but also intracellularly for ricin cytotoxicity.

Solis *et al* showed that the binding properties of ricin could be studied without undue concern about its safety in handling by modifying its carboxyl groups.[27] The modified toxin was 90-fold less toxic than native ricin, but the strength and specificity of the carbohydrate- binding ability of the lectin were substantially retained.

ENDOCYTOSIS AND INTRACELLULAR TRANSPORT

Magnusson and colleagues have studied the pathways of ricin endocytosis extensively in rat liver endothelial cells, paying particular attention to mannose receptors as mediators of the internalisation, since both the A-chain and the B-chain are glycoproteins containing mannose-rich oligosaccharides[22,28,29] Previous research had concentrated on the galactosyl residues. Approaches adopted by these authors include binding and uptake of radiolabelled ricin, inhibition of protein synthesis, sub- cellular fractionation, retroendocytosis (i.e. the reverse pathway of endocytosis), immunocytochemistry and the role of other liver cell types. Differences were observed between the galactose and mannose internalisation pathways in the transport from endosomes and lysosomes, and these were related to the different stabilities of the two binding mechanisms at endosomal pH. In particular, the binding of ricin to galactosyl residues displays unusual stability at low pH,'0 although the optimum pH for ricin toxicity is between 7 and 8. Mannose receptors are more efficient at accumulating an inhibitory intra- cellular concentration of ricin than galactosyl residues, and the onset of toxicity was accordingly more rapid after internalisation via mannose receptors. Several sub-cellular compartments were found to be involved in internalisation, including coated pits, coated vesicles, Golgi apparatus and different types of endosomes and lysosomes. The role of the Golgi-structure is controversial[30] and has been studied by Sandvig et al, Ryser *et al*, and Bau and Draper (1993).[31.32.33]

Oda and Wu reported that the antibiotic cerulenin reduced the internalisation of ricin, but not it's binding to cell-surface receptors, in a mutant of monkey kidney cells; however, it had no effect on the parent cells[21]. Naseem and Pace reported that fluocinolone (an anti-inflammatory glucocorticoid) increased ricin-induced inhibition of protein synthesis by increasing the binding efficiency and

internalisation of ricin.[34] On the other hand, a nonsteroidal anti-inflammatory drug (indomethacin) protected macrophage cell lines against ricin. Wales et al provided evidence in favour of their proposal that the pathway of ricin to the interior of the target cell is the exact reverse of the secretory path for toxins, via the endoplasmic reticulum[49]

TRANSLOCATION TO THE CYTOSOL

The endoplasmic reticulum membrane may be the principle target for translocation of ricin and other toxins.[35 49]

Hegde *et al* observed that inhibition of protein synthesis in cells by toxins, as measured by incorporation of radiolabelled amino acid, was preceded by a concentration-dependent lag phase,[15.20] which they interpreted as the time the toxin takes to travel through the cytoplasmic compartments. Fluorescence techniques were used with rat thymocytes to compare the kinetics of intoxication and inter-subunit disulphide reduction by abrin variants and ricin. The results indicated that the binding and internalisation efficiencies of the various toxins were the same, and that the observed differences in the dose-dependent lag time were causally related to the proposed intracellular processing event. Other kinetic studies also suggest that the rate-limiting step in cytotoxicity under most circumstances is A-chain translocation. The B-chain appears to facilitate the transfer of the A-chain to the cytosol by insertion of hydrophobic regions into the membranes of endosomes forming a pore through which the A-chain may be delivered to the cytosol.[15 36] The toxic effect of ricin can be countered by antitoxins only during the first 30 min of a 6h lag at 20°C, indicating that during the remaining 5.5h the toxin is not exposed on the outside of cells[7]. Madan and Ghosh studied the ability of monensin, a carboxylic ionophore which is known to raise intravesicular pH, to reduce the lag period and enhance the cytotoxicity of ricin.[37] Although this study was aimed at increasing the efficiency of ricin as an anti-tumour agent, monensin may be a useful tool for mechanistic studies.

ENZYMATIC INACTIVATION

Endo *et al* found that the 28S rRNA is the only target RNA for ricin and also for the ricin-related toxins abrin and modeccin.[38] These other toxins also acted at a site close to the a-sarcin cleavage site. Other results suggest an important role of ribosomal proteins in inducing and maintaining the secondary structure recognised by ricin A-chain, including the fact that denatured 28S rRNA was not modified by ricin A-chain, which does not act as a ribonuclease but as an N-glycosidase.[38] Endo *et al* proposed that elongation factors 1 and 2, directly or indirectly via hydrolysis of GTP, initiate the reversible transition or switch in rRNA structure that propels translocation, by means of a primitive allosteric transition. Depurination at A4324 in 28S rRNA by ricin or cleavage at G4325 by a-sarcin might abolish the capacity to switch structure reversibly and account in this way for the catastrophic effect of the toxins on ribosome function. In this study, Endo et al used mutants of a synthetic oligoribonucleotide, as a model of the ricin substrate, to study the kinetics of the enzymatic inactivation. This 35mer has the sequence and the secondary structure of the ricin region of 28S rRNA.

There is evidence that ricin produces an oxidative stress, mediated by free radicals, in the liver after injection intraperitoneally in mice [5]. The lipid peroxidation which was observed in this study could be either a cause or an effect of the reactions producing toxicity. Site-directed mutagenesis has shown the importance of the carboxylate function at position 177 of ricin (Giu 177) for its enzymatic activity [27]. The same technique, coupled with X-ray diffraction, was used by Kim and Roberts to show that Arg 180 is involved more in transition state stabilisation than in substrate binding.[30]

Hassoun *et al* examined specific glycosidase inhibitors as possible chemoprotectants against ricin[40] Six compounds, believed to form covalent enzyme linkages, were tested in an in vitro system (release of lactate dehydrogenase and aspartate aminotransferase from cultured macrophage cells). Two were found to exhibit significant activity against ricin toxicity; these were N-bromoacetyl-a-D-galactopyranosylamine and the b isomer. It is plausible that these compounds inhibit ricin activity after cellular internalisation of the toxin: however, it is also possible that they competitively bind to the cell membrane galactose binding site to act as a false receptor and prevent ricin transport into the cytoplasm. Note that these compounds exhibited some cytotoxicity of their own, i.e. in the absence of ricin.

IMMUNISATION

Foxwell *et al* prepared antibodies to ricin and it's separate A- and B-chains and evaluated them in a cell-free assay; against lymphoma cells, in haemagglutination studies and in vivo (mice)[41]. The antibodies were effective in vivo (as well as in the other systems), even when given up to 640min after subcutaneous ricin or up to 20-40min after intravenous ricin. The use of antibodies as a treatment in cases of accidental ricin intoxication may therefore be feasible, depending on the amount of ricin absorbed and the route of entry. Houston also reported that antibodies could protect mice against ricin if given after exposure (up to 3h in this case).[42] Prophylaxis against ricin in mice using monoclonal antibodies (not all that successful) has been reported by Chanh *et al*[43]. Other studies with antibodies to ricin are described by Colombatti *et al*[44].

Hewetson *et al* established the feasibility of protecting mice against inhaled ricin by both active and passive immunization[6]. It is interesting that up to 1,000- fold more anti-ricin Immunoglobulin G intravenously was required to protect mice against aerosolised ricin than against intravenous ricin. These results suggest a different mechanism of action of ricin administered by inhalation, the inability of antibody to enter the alveolar spaces at sufficient concentrations or the requirement of a different class of antibody (e.g. IgA) to protect against exposure at mucosal surfaces.

Lemley and Wright found that mice passively immunised by a protective, anti-ricin A-chain monoclonal antibody, then challenged intravenously with ricin, were protected from a subsequent ricin challenge, and were actively immunised[45]. They concluded that the monoclonal antibody neutralised toxicity of ricin immunogen and that active immunisation was achieved with very low antigen load (-0.5 mg/mouse). As a result of its extreme in vivo toxicity, however, ricin cannot be used as the immunogen to elicit protective immunity[46]. Chanh and Hewetson achieved anti-ricin immune responses with mouse and rabbit polyclonal anti-idiotypic antibodies, raised from protein G-purified goat anti-ricin Immunoglobulin G 46 A ricin toxoid has been developed which when used as vaccine protects against toxin given by inhalation[2] and intravenously in mice[47].

As mentioned above, ricin binds to galactose, and if a polysaccharide of this sugar (agar is a polymer of galactose having sulfhydryl groups) can be introduced into the cell, e.g. by liposome therapy, it may have some therapeutic value, as might inactivating monoclonal antibody that binds to ricin's galactose-binding domain. As there is little effective therapy once the damage has been done, the best option may be to protect troops with vaccines or protective human monoclonals.[2]

THERAPEUTIC APPLICATIONS OF RICIN

Immunotoxins are chimeric, antineoplastic molecules constructed by covalently conjugating monoclonal antibodies to plant or bacterial toxins[36]. The antibody moiety allows specific targeting of the immunotoxin to tumour-associated antigens, while the toxin moiety is responsible for cell killing by

irreversible inactivation of protein synthesis. In the case of ricin, monoclonal antibodies linked to ricin A-chain can substitute for the ricin B-chain binding.[15] The potent protein synthesis inhibitory activity of ricin has been utilised as the cytotoxic effector moiety of many immunotoxins with potential use for cancer therapy. The limitation of antibody-ricin conjugates in vivo is that immunotoxins lacking the ricin B subunit have much lower toxicity to tumour cells (because of the slow rate of cell killing),[15] and immunotoxins containing the ricin B-chain can have significant non-target cell toxicity. Attempts to circumvent non-specific cell attachment of ricin via ricin B chain immunotoxin have included treatment of cells in the presence of high concentrations of lactose, and decreasing galactose binding by steric hindrance achieved by cross-linking ricin to the antibody[22]. Ricin B-chain increases the specific toxicity of antibody-ricin A-chain conjugates by increasing the rate of entry of ricin A-chain into the cytosol. Further, ricin B-chain linked to antibodies will potentiate the toxicity of ricin A-chain linked to antibodies.

In vitro studies have shown that ricin A-chain carried by red blood cells is able to kill efficiently erythrophagocytic cells having ingested such a carrier. Ricin A-chain targeted to the erythrophagocytic cells does not seem to significantly modify the in vivo phagocytic activity and the antibody response against a heterologous cellular antigen, i.e. there is an absence of effects on the immune system. The efficiency of this carrier devoid of side effects would be encouraging data for its use in therapeutic applications[48].

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