Retrospective Identification of Ricin in Animal Tissues Following Administration by Pulmonary and Oral Routes

David Leslie Cook, Jonathan David and Gareth David Griffiths
Biology, Biomedical Sciences, Dstl, Porton Down
Salisbury, Wiltshire, SP4 0JQ, UK

Gareth D Griffiths contact details:

- mailing address Immunotoxicology, Toxins and Pathology Team, Biology, Biomedical Sciences, Dstl, Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK.
- Tel: 01980 613367; Fax: 01980 613742
- E-mail: gdgriffiths@dstl.gov.uk

This work was funded by the UK Department of Health

SUMMARY

Detection of a toxin in tissue samples may be important for medico-legal and forensic purposes in demonstrating proof of exposure. It may also be important in informing medical teams if and when to administer therapies. Ricin is a toxin which could be used by terrorists and work is well underway at Dstl Porton Down, on the production and optimisation of an antitoxin to be used therapeutically to mitigate the effects of ricin poisoning. It is undesirable to administer quantities of a foreign protein, such as an immunoglobulin, unnecessarily to people and thus it is important to identify those who require treatment. It is also important to understand how easily detection of exposure may be achieved in a relatively short time frame, because once ricin is internalised into cells and tissues, treatment with an antitoxin is likely to become ineffectual. A pilot study was, therefore, set up to explore appropriate animal tissue samples for the identification and quantification of ricin over a time period after exposure by pulmonary or oral routes. A previously characterised amplified ELISA for ricin (sensitivity limit approximately 200 pg.ml^{-1}) was employed to quantify ricin following a novel recovery method from selected tissues. Tissue samples from rats dosed by pulmonary instillation or orally with ricin were homogenised and treated with an elution buffer to extract ricin. Ricin was recovered from tissues post pulmonary or oral challenge using clinically acceptable sampling methods, with promise in terms of diagnosis for the timely implementation of therapy. The toxin was detected and quantified using the ELISA in conjunction with pure ricin standards. Extracts from tissues sampled, including lung, blood, liver and spleen tested positive for ricin, with maximum yields in lung associated fractions after pulmonary dosing and liver tissue after oral administration. This indicates the potential of lavage and blood sampling for timely diagnosis of ricin poisoning by pulmonary and oral routes respectively. In addition to offering the selection of appropriate samples for making an early diagnosis of ricin poisoning, this method would be useful for the identification of ricin or closely related proteins (together, ricin 'equivalents') in tissues for forensic purposes in a medico-legal setting.

KEYWORDS: crude ricin; oral route; pulmonary route; instillation; ELISA; retrospective identification; quantification; ricin equivalent protein; diagnosis.

Cook, D.L.; David, J.; Griffiths, G.D. (2007) Retrospective Identification of Ricin in Animal Tissues Following Administration by Pulmonary and Oral Routes. In *Defence against the Effects of Chemical Hazards: Toxicology, Diagnosis and Medical Countermeasures* (pp. 13-1 – 13-16). Meeting Proceedings RTO-MP-HFM-149, Paper 13. Neuilly-sur-Seine, France: RTO. Available from: http://www.rto.nato.int.

Note: Aspects of this study were prepared and accepted for publication elsewhere

Cook, D L, David J and Griffiths G D. Retrospective identification of ricin in animal tissues following administration by pulmonary and oral routes. Toxicology 223, (2006), 61-70

Introduction

Ricin is a major protein produced in the seeds of the castor oil plant, *Ricinus communis* and represents about 2% of the total weight of a seed. Castor oil comprises the greatest component by weight (60% by weight of each seed) of *Ricinus* seeds. Other soluble proteins present in the seeds include *Ricinus* agglutinin, virtually a dimer of ricin but much less toxic, as well as many smaller proteins.

Ricin is a potent toxin, which kills eukaryotic cells by inhibiting protein synthesis. It is one of a class of toxins known as ribosome-inactivating proteins (RIPs; Barbieri et al., 1993). These proteins interact with a discrete part of the 60 S subunit of cellular ribosomal RNA and cleave one nucleotide base, adenine 4324. This action prevents the binding of elongation factor 2 and arrests protein synthesis (Jiminez and Vasquez, 1985). RIPs exist as type 1 or type 2, based upon their structure, being either monomeric or dimeric, respectively. Ricin is an example of a type 2 RIP. Others include abrin, modeccin, volkensin and viscum album toxin. (Stirpe et al., 1992)

Type 1 RIPs are produced by many plants and consist of an A chain which has N-glycosidase activity, capable of cleaving ribosomal RNA. Type 2 RIPs consist of the A chain but additionally, this is bound through a disulphide bond to a B chain. The B chain has lectin properties, binding galactose or N-acetyl-galactosamine sugars (Olsnes and Pihl, 1982). By virtue of this lectin domain, type 2 RIPs are able to bind to appropriately-glycosylated cell surface proteins or lipids and become internalised by endocytosis. During this process the toxin becomes dissociated from the membrane and the A and B chains become separated through hydrolysis of the connecting disulphide bond when the endocytic vesicle is acidified. A proportion of the internalised ribotoxin is channelled via the trans-Golgi to the cytoplasm, where it gains access to the ribosomes. A more detailed description of this process and the subsequent cleavage of adenine is given in the review by Lord et al (1994).

In view of the relative ease with which a crude toxic extract rich in ricin may be prepared from castor beans using simple technology, it is considered to be a realistic terrorist chemical weapon. Indeed, this toxin has been of concern to the military for several decades and considerable effort has been made in the U.K. and the U.S. to produce medical countermeasures effective in neutralising and mitigating the harmful effects of ricin. These have included the the development of vaccines against ricin (Griffiths et al 1995, 1997, 1998; Wannemacher et al 2006) and latterly, the preparation and optimisation of an antitoxin. Emergency medical service providers need information on the properties of the toxin (Bradberry et al., 2003) and most importantly, need to have the capability for the early diagnosis of exposure to ricin. This would allow triage of victims who had received ricin and inform medical care providers of the requirement to administer ricin antitoxin. Effecting an early diagnosis of exposure to ricin will maximise chances of survival using the window available for therapeutic intervention. In order to be in a position to do this, tissue samples must be selected over an appropriate time course in a suitable animal model so that evidence of exposure to ricin may be gained. Most commonly, antibody-based technologies such as enzyme linked immunoadsorption assay (ELISA) are sufficiently sensitive to exploit for the diagnosis of the presence of ricin in tissue samples. A positive signal in such an assay could result from the presence of ricin toxin, *Ricinus* agglutinin, which shares many epitopes with ricin or breakdown products of either protein. This is a property of the ricin-specific antibodies used for the assay. In addition to early

notification of exposure, the retrospective identification of ricin poisoning could also be important for medico-legal reasons.

In view of the potency of ricin, the method must be very sensitive. An ELISA was developed (Leith et al., 1988), which could detect ricin in body tissues to a limit sensitivity of about 200 $pg.ml^{-1}$ following intramuscular injection. The detection limit is partly a function of the quality and properties of the antibodies used in the system but also of the efficacy with which ricin is extracted from tissue samples and made available to the assay. The present study describes a current version of this tissue treatment and ELISA developed at Dstl, Porton Down. Despite employing different capture and reporter antibodies, the procedure was found to have the same limit of detection as the original assay. Here, this adapted ELISA was applied to tissue samples following intoxication of a small number of rodents with a crude preparation of ricin by pulmonary and oral routes. Crude ricin was selected as a likely candidate for offensive use by an aggressor.

While information on inhalational exposure is wholly reliant upon animal studies, there have been many accidental or intentional poisonings by oral administration of ricin, examples of which have been reported (Smith et al., 1985; Aplin and Eliseo, 1998).

The methods discussed enabled both the novel recovery and identification of ricin, *Ricinus* agglutinin or breakdown products in several tissue samples. For this reason, the quantification of ricin in representative samples, in conjunction with a standard curve using purified ricin in the ELISA are expressed as ricin 'equivalent' protein. Purified ricin was selected for production of the standard curve since it could be readily quantified for use as a standard, whereas crude ricin could not. The method offers scope for early screening of potential victims of ricin exposure by oral or pulmonary routes, using appropriate, acceptable clinical sampling techniques, with a view to implementation of therapeutic strategies. Since the current approach recognises several proteins present in the castor oil plant it could not be used to give a true estimate of dosage received but rather, to confirm that exposure to a crude preparation of ricin had occurred. Without the extraction of toxin from tissue samples from the victim and its subsequent analysis, it would be difficult to be sure whether challenge had been with a purified or crude ricin sample.

Methods

Animals

Rats used in this study were male Porton Wistar rats of bodyweight approximately 250g. Animals were habituated to the experimental animal unit for 1 week prior to use in the study. All work was conducted in accordance with the Animal (Scientific Procedures) Act, 1986.

Ricin

Pure ricin toxin was prepared in-house from seeds of *Ricinis communis* var. *zanzibariensis* as described previously (Griffiths et al., 1995). Briefly, following homogenisation of the seeds, ricin toxin was extracted from the clarified supernate by ammonium sulphate precipitation followed by affinity and molecular sieving chromatography by Fast Protein Liquid Chromatography (FPLC, Pharmacia). The toxin preparation appeared as a single band of molecular weight approximately 65kDa, on a silver-stained non-reducing polyacrylamide gel by electrophoresis.

Ricin used in the animal dosing study was a centrifuged crude, defatted preparation made from *Ricinus communis var.zanzibariensis* castor bean seeds. Lipid extraction was carried out using multiple acetone washes and the resulting dried powder solubilised in phosphate buffered saline (PBS). The preparation was subsequently centrifuged at 11,000 x g for 30 seconds to remove precipitated material. The resultant crude preparation contained approximately 50% ricin, based upon densitometric analysis of an SDS-polyacrylamide gel. The protein concentration of the whole crude ricin preparation was determined to contain 5 mg ricin per ml solution (bicinconinic acid method; Kopferschmitt et al., 1983). Dosing was based upon the concentration of ricin determined by this procedure.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The crude ricin sample was examined using electrophoresis performed in gradient gels (8-25%) using a Pharmacia Phastsystem electrophoresis system. The gels were stained with Coomassie stain (Sigma-Aldrich: Brilliant Blue R B6529 Lot 49H927J).

Densitometry

Electrophoresis gels were scanned (Biorad GS-800 calibrated densitometer) and analysed using a software programme designed for the analysis of single dimension electrophoresis gels (Biorad Quantity One). Band density was corrected for non-specific background staining and relative quantity expressed as a percentage of total proteins in the sample.

Animal Procedures

Pulmonary dosing

Rats were dosed into the lungs via the trachea using instillation. Animals were anaesthetized using halothane in an anaesthetic box. Anaesthetized animals were taken from the box supported at an angle of approximately 50 degrees with head uppermost and dosed from a 1 ml syringe fitted with a blunt-ended cannula. The tip of the cannula was inserted gently through the pharynx into the trachea. The correct location of the cannula was confirmed, in part, by feeling the cartilage rings of the trachea as the tip was carefully moved up and down. A small volume (0.1 ml) of either crude ricin/PBS solution (0.8 µg ricin.0.1 ml^{-1}, approximate LD_{30} dose) or PBS (vehicle) was expelled into the trachea and the cannula removed. The rat was held in an upright position until it regained consciousness (30 seconds to 1 minute), during which time it was possible to hear the rales caused by successful fluid instillation into the lungs. Once conscious, all animals moved freely around the cage without any signs of distress.

Oral dosing

Rats were held by the scruff in a supine position, supported on the palm of the hand. Soft catheter tubing was fitted to a 2 ml syringe, loaded with 1 ml of ricin/PBS solution or PBS (vehicle). Rats were dosed with crude ricin at 2 mg ricin in 1 ml (8 $mg.kg^{-1}$ bodyweight, approximate LD_{50}). The tubing was gently introduced through the mouth of the animal, advanced carefully along the back of the buccal cavity. Once swallowed, the tube was introduced into the oesophagus. The sample was expelled slowly and steadily. The catheter tubing was then withdrawn and the rat held for a further period of a minute or so, to closely monitor its wellbeing.

Culling and tissue sampling

Pulmonary dosed group

Rats were culled at 24 and 48 hours following the pulmonary challenge with ricin by lethal intraperitoneal injection (150 $mg.kg^{-1}$bodyweight) with sodium pentobarbitone (Euthatal). The thorax was opened, the sternum and part of the rib cage was removed to expose the lungs. The trachea was opened with the tip of sharp pointed scissors and a small bore soft catheter tube introduced and tied in place with surgical thread. The tubing was fitted to a syringe via 3-way tap and 5 ml of sterile PBS was introduced into the lungs, to fully inflate and wash them out. The 3-way tap was adjusted to allow the lung wash fluid to be withdrawn into a separate syringe. This was held on ice whilst the process was repeated twice more. Lung wash lavage fluids from successive washes in each single animal were pooled and centrifuged to recover pulmonary cells (mainly macrophages and any inflammatory cells present). The cell pellets were resuspended with 1 ml of elution buffer, mixed on a vortex mixer and rotated at 4^0C overnight. Following centrifugation (1800 x g 15 mins), the supernatants were removed and stored frozen (-80^0C) until analysed. Elution buffer was made up in PBS and had the following composition: bovine serum albumin 0.1 % (w/v), Triton X-100 1.0 % (v/v) and galactose 0.25M. The remaining lung wash fluid was concentrated to 1 ml using Vivapore 5, solvent absorption concentrators having polyethersulfone membranes with molecular weight cut off around 7.5 KDa (Z61, 505-6 SIGMA).

Lung tissue was also removed, weighed, wrapped in catering tin foil and snap frozen by immersion in liquid nitrogen. The wrapped tissue was placed on a metal block and smashed using a hammer. The smashed tissue was transferred into a pre-determined quantity of elution buffer on ice, homogenised using an Ultra-Turrax T8 (IKA-Werke GmbH & Co. KG) with mini-probe (S8-5G 5mm dispersing element) on maximum setting until sample appeared uniform. Tissues were homogenised in volumes of elution buffer according to the following ratios, all expressed per 100 mg of tissue: liver 0.1ml; spleen 0.15 ml; GI tract 0.15 ml; kidney 0.15 ml; lung 0.1 ml; lung wash cells: 1 ml elution buffer to cell pellet.

Orally dosed group

After oral dosing with crude ricin at 8 $mg.kg.^{-1}$ (~ LD_{50}) rats died much sooner than expected and the animals looked unwell at around 24 hours; 2 of 3 dosed initially, died by 26 hours. The remaining animals were also dosed at 8 $mg.kg^{-1}$ but were culled using Euthatal at 24 hours. Blood (2 ml) was sampled by cardiac puncture into heparinised tubes and placed on a roller mixer for about 10 minutes. Plasma was separated from cells by centrifugation for 10 minutes at about 1000 x g. Supernatant (plasma) was removed, mixed with an equal volume of elution buffer and stored frozen (as above). Blood cells (from the 2 ml sample) were mixed with red cell lysis solution, ammonium chloride (PharMLyse, BD Biosciences). PharMLyse stock concentrate was diluted 10 fold to working dilution (x1) and 15 ml were added to the cell pellets. After gently shaking to resuspend cells, samples were rotated on a mixer for 10 minutes at room temperature to allow red cell lysis. In addition to blood, 5 tissues were removed. These included: stomach plus proximal duodenum, mesenteric lymph nodes, liver and spleen. These solid tissues were snap frozen, smashed and ricin extracted by homogenisation in elution buffer, as described above. Insoluble tissue debris was removed by centrifugation (1800 x g; 15 minutes). Clarified supernatant extracts were re-frozen at –80^0C until the assay for ricin content was performed.

ELISA

The ELISA was an antigen capture sandwich procedure, amplified by using biotinylated donkey antibody reagent against the ricin detecting secondary antibodies and streptavidin peroxidase as a colour generating

system. The capture antibody was monoclonal (mouse) R48 with ricin A-chain specificity and the second detection antibody was sheep anti-ricin (IgG fraction). Both were supplied by Detection Department, Dstl, Porton Down. Biotinylated donkey anti-sheep IgG and streptavidin peroxidase were purchased from Sigma-Aldrich. All samples, pure ricin standards and controls were run in replicates of 4 on the assay plates.

Ninety-six well microtitre plates (Corning # 3595 – Costar polystyrene flat bottom plates) were coated with R48 capture antibody at 10 $\mu g.ml^{-1}$ (100 µl per well) in PBS and incubated at 4^0C overnight. The outer wells around the plate were left empty, using the inner 60 wells for assay, because of noted edge effects. The following day the wells were emptied, tapped out onto absorbent paper and the wells blocked by the addition of 100 µl of 1% Blotto (non-fat dry milk powder) in PBS, at 30^0C for 1 hour. Blocker was tipped out of the plates, which were then tapped out as above, and standard purified ricin solutions (0.2-2$ng.ml^{-1}$), elution buffer alone or samples (undiluted or at dilutions in elution buffer) were added to the wells. Plates were incubated for 1 hour at 37^0C and then emptied (as above) and sheep anti-ricin antibody reagent was added at a dilution of 10 $\mu g.ml^{-1}$ in antibody dilution buffer (1% Blotto in PBS) for 1 hour at 37^0C. Plates were emptied as above, washed 3 times with wash buffer (0.05% v/v Tween 20 in PBS) using an automated plate washer (Thermo Labsystems Ultrawash Plus). Biotinylated donkey anti-sheep IgG antibody was added (100 µl per well) at a dilution of 1:100 in antibody dilution buffer (control wells were set up in which this reagent was omitted, to determine non-specific signal generation). Plates were incubated and then emptied as before, and finally washed as described above. Streptavidin peroxidase reagent was added (100 µl per well) at a concentration of 1.0 $\mu g.ml^{-1}$ in dilution buffer and plates were incubated as above. After emptying, plates were washed 5 times using the automated washed and colour reagent was then applied (100 µl per well) to the wells. This consisted of ABTS (2,2'-azino-bis(3-ethybenzthiazoline-6-sulphonic) acid) and H_2O_2 (0.01%) in citrate phosphate buffer. Plates were incubated at 37°C and absorbances were read (Thermo Labsystems Multiskan Ascent) every 2 or 3 minutes (414 nm) until the control wells reached an absorbance of about 0.1 when final readings were taken.

Calculation of ricin in samples

Average values were determined of the final absorbances of pure ricin standards, tissue eluates from ricin-dosed rats, vehicle controls and reagent controls. Mean reagent control absorbances were subtracted from the other assay absorbances. Quantitative data were prepared using purified ricin standards in each assay plate to prepare a standard curve (0.2-2$ng.ml^{-1}$). The vehicle control absorbance data were subtracted from corresponding ricin-positive sample absorbances and these processed data were used to interpret the ricin 'equivalent' concentrations in samples from the standard curve. This was performed using the equation of the standard curve. In practice, all samples were initially assayed undiluted but were subsequently re-assayed at an appropriate dilution (given with the results) to correlate with the standard curve. Since dosing employed crude ricin, some signal in the positive samples may have resulted from *Ricinus* agglutinin or breakdown products, in addition to ricin toxin, so that only a ricin 'equivalent' concentration may be inferred in samples.

Results

Analysis of crude ricin preparation

Crude ricin was found to have a total protein concentration of 10 $mg.ml^{-1}$ by BCA assay. Using SDS-PAGE at least 5 protein bands were contained within the centrifuged crude ricin preparation of which the major component was ricin which was found by densitometry to represent about 50 % of the total protein (data not shown). Thus the crude ricin preparation was, in this way, determined to contain 5 mg ricin toxin

per ml solution. The position of ricin was inferred by running a sample of pure ricin in the adjacent gel lane. Small proteins and peptides of molecular mass below 14.4 KDa were the next most abundant group (13.1%), followed by *Ricinus* agglutinin which represented about 9% of the total protein. Minor protein bands represented about 3% of the total protein.

Pulmonary dosed group

All animals survived to the intended cull times of 24 and 48 hours after dosing with 0.8µg crude ricin. The lung tissue homogenates from the ricin-treated rats were observed to have 'coagulated', compared with the tissue from vehicle controls, where tissue fragments were not closely associated with each other and moved more freely under agitation.

ELISA results on lung tissue homogenate, lung wash fluid and cells (at 24 hours) isolated from the lung wash fluid were all positive for ricin 'equivalent' proteins, over and above vehicle tissue controls. The absorbances (414nm) measured by ELISA for ricin 'equivalent' proteins showed lung wash samples to be the highest in magnitude at 24 hours with the cellular component of the lung wash detectable at low levels. 48 hours after dosing the ricin 'equivalent' level in the lung wash cell fraction was no longer detectable. However, the tissue sample was markedly increased and the highest of the three samples at this later time-point.

Absorbance values indicative of the presence of ricin and related proteins, were all significantly higher (except lung wash cells at 48 hours) than the corresponding tissue eluate control data. Examination of the vehicle and ricin-treated group data showed large differences in variance and, because of relatively small sample size, it was difficult to confirm that the data were normally distributed. For these reasons, the data were analysed using the non-parametric Mann-Whitney two sample rank test at 24 and 48 hours, using a significance level of p=0.02.

Statistical analysis of the data gave the following outcomes (98% confidence intervals): Tissues in which ricin 'equivalent' assays in ricin-treated and corresponding vehicle-treated tissue samples were significantly different included lung wash fluid at 24hours (CI 0.771, 2.565) and at 48hours (CI 0.563, 2.241), lung wash cells at 24h (CI 0.219, 1.393) and lung tissue at 24h (CI 0.802, 2.638) and at 48h (CI 0.848, 2.308).

The line of best fit through the purified ricin standard sample data (Figure 1) showed fair correlation (R^2 = 0.9125). Ricin 'equivalent' concentrations in tissue samples were determined using the standard curve. In order to determine ricin 'equivalent' content of tissue samples, averaged sample replicate absorbance data were corrected for non-specific signal and the signal generated by the corresponding tissue vehicle controls. The data were also corrected for sample dilution factor and total ricin 'equivalent' per tissue sample was determined by incorporating the volume of the total elution buffer added to each tissue. For normalisation of the data the concentration of ricin 'equivalent' per gram of tissue was deduced and finally, the total 'equivalent' toxin content recovered was expressed as a percentage of the original dose (800ng). These data for the lung-based study are summarised in Tables 1a and 1b.

Pulmonary lung wash fluid was found to contain a mean value of 40.75 ng of ricin 'equivalent' at 24 hours (Table 1a). By 48 hours, however, lung wash fluid ricin 'equivalent' content had fallen to a mean value of

1.25 ng (Table 1b). Lung fluid wash cells contained an average of 3.87 ng of associated ricin 'equivalent' protein at 24 hours but none was measured at 48 hours. Lung tissue was found to contain 11.45 ng ricin 'equivalent' protein at 24 hours but this amount had almost quadrupled to 40.89 ng by 48 hours.

Orally dosed group

A limited previous study at Dstl on oral toxicity of ricin in the mouse indicated that the LD_{50} was likely to fall in the range 1-10 mg per kg, some 3 orders of magnitude lower potency by this route. The oral dose of crude ricin used in this rat study reflected this guideline and was based upon the ricin content of the sample, determined from densitometric analysis of the electrophoretic gel of the crude ricin . Three rats were administered 8 mg per kg bodyweight of crude ricin and when observed 24 hours later, all looked unwell and 2 of them died at 26 hours. Blood samples were taken, in addition to a full range of tissues. It was decided to continue with the same dose for the rest of this study but to terminate at 24 hours.

Examination of the vehicle and ricin-treated group data showed large differences in variance and further, because of relatively small sample size, it was difficult to determine normality. For these reasons, non-parametric analysis of the data was performed, using a Mann-Whitney two sample rank test, using a rejection level of p=0.02, because of the number of comparisons.

Ricin 'equivalent' proteins were detected in blood analysis at the 24-hour observation time associated with the cellular content of the blood rather than the plasma, which failed to give a statistically significant signal above background in the ELISA (data not shown). Liver tissue accumulated a small quantity of ricin 'equivalent' protein but there was more in the GI tract (although not significantly different from vehicle, see below) and in the spleen. The kidney analysis (not statistically different from vehicle, see below) showed similar amounts of ricin 'equivalent' protein to the GI tract. Statistical analysis of the data gave the following outcomes (98% confidence intervals): tissues in which ricin assays in ricin-treated and corresponding vehicle-treated tissue samples were significantly different included blood cells (CI –1.333, -0.055), liver (CI –0.805, -0.0079) and spleen (CI –2.722, -0.167).

The ricin 'equivalent' content of individual tissue samples was interpreted using absorbances determined in the ELISA at appropriate sample dilutions from the pure ricin standard curve (Figure 1), run in the same plates at the same times. Quantitative analysis of data for ricin in the ricin-positive tissues associated with oral dosing (Table 1c), revealed that a only small quantity (1.4 ng) of the original 2 mg dose was associated with the cellular fraction of the blood. Most ricin 'equivalent' (54 ng) was found in liver tissue (Table 1c). Spleen tissue contained the next highest quantity of ricin 'equivalent' (17.3 ng), followed by GI tract tissue and kidney, which contained very similar quantities of around 7 ng. The data were also expressed as toxin per gram of tissue (deduced from extracted tissue weights) and as percentage recovered from the original dose (~2mg per animal).

Mesenteric lymph nodes (MLN) were collected from one rat, which had swollen, dark red, reactive MLN but the ELISA was unsatisfactory and no sample was available for a repeat assay.

Discussion

The method of extraction of ricin from tissues and the ELISA was originally developed to detect and quantify ricin in various tissues following intramuscular dosing with the pure toxin (Leith et al., 1988).

This present study has shown that it is feasible to use this approach to detect the presence of ricin, including any closely related proteins such as *Ricinus* agglutinin and possibly any breakdown products, in realistic samples taken from living victims of poisoning, after moderate doses of this toxin, by pulmonary or oral routes. Ricin and closely related proteins targeted by this approach have been referred to as ricin 'equivalent' proteins.

The method of exposure by the pulmonary route was by intratracheal instillation of ~ LD_{30} of ricin rather than by aerosol inhalation. This route has been criticised following validation as a model for aerosol inhalation, particularly because of differences in the final distribution of instillate compared with inhaled material (Vogel et al., 1996). Whilst understanding the limits of this method in terms of a realistic exposure, the fact that the toxin could readily be detected and measured, mainly in association with lung lavage fluid, bodes well for chances of doing the same following inhalation exposure to ricin. This is assisted by the fact that broncho-alveolar lavage is a recognised clinical procedure and could be applied to living patients who were suspected of ricin inhalation exposure. Thus instillation served to indicate the possible merit of lung wash fluid and contained cells to signal ricin following inhalation exposure.

Previous studies have indicated that distribution of ricin in murine tissue is maximal prior to 24 and 48 hours (Doebler et al., 1995). However, for diagnosis of exposure, it is unlikely that clinical sampling will occur instantaneously and thus a need to detect ricin is required for times which may be later than those associated with maximal distribution, whilst still within the time-frame for intervention. Beyond these timepoints following superlethal exposure death would likely have occurred.

At the 24 hour time point, ricin 'equivalent' proteins were found to associate with lavage fluid and, to a small extent, with cells in this fraction (presumably macrophages and neutrophils). This fits the electron microscopy study of Brown and White (Brown and White, 1997) in which inhaled ricin was found to have an early effect on pulmonary macrophages. By the 48 hour time period, ricin 'equivalent' proteins were present in lavage fluid but mostly within lung tissue itself. Receptors for ricinus lectins have been identified on lung epithelial cells (Dobbs, 1982; Taatjes et al., 1990), so it is perhaps not surprising that ricin 'equivalents' were found to have transferred from lavage to concentrate within lung tissue at the later time period. Lung lavage would, therefore, appear to be a good source of ricin for diagnostic purposes. Lung tissue could also be used but due to the invasive nature of tissue sampling perhaps would be more suited to *post-mortem* examination.

It is interesting to note that lung tissue homogenates from ricin-dosed animals had the appearance of 'coagulating' in contrast to the saline-treated control counterparts, which produced tissue fragments which were freely mobile during agitation. This phenomenon was presumably due to the lung cell receptor-binding properties of the crude ricin challenge which would contain ricin and the divalent lectin *Ricinus* agglutinin. Following inhalation of ricin the permeability of the pulmonary endothelium increases as a result of localised inflammation. This increase in permeability allows the migration of inflammatory cells into the lung and consequently may allow the translocation of ricin into the blood. In previous inhalation studies of ricin in the rat, we have not found evidence for ricin leaving the lung compartment to cause peripheral damage (Griffiths et al., 1995), and as such any ricin present in blood through this mechanism would be minimal. It is, however, possible that small quantities of ricin may be swallowed in mucus and saliva from the nasal (and buccal) cavities. Therefore for these reasons blood was not analysed for the presence of ricin following these pulmonary instillation studies.

The toxicity of ricin by the oral route is several orders lower than by pulmonary or injected routes (Gill, 1982), partly because of degradation in the gastrointestinal tract but also because of the poor uptake and transfer of ricin into the blood by the healthy tract. It appears that it is only when the intestinal barriers become more permeable through the establishment of a robust inflammatory state or perhaps through tissue damage, that ricin passes into the bloodstream and hence, is transferred to other remote tissues.

The LD_{50} of ricin in the rat was exceeded in the oral dosing study. Since it was not possible to undertake dose-lethality analyses during the course of the work, ricin was orally dosed using toxicity data generated in another rodent species, the mouse. It was found, however, that this bodyweight-based dose (approximately 20 $mg.kg^{-1}$ bodyweight) was more toxic to the rat and lower doses were subsequently administered, but remained in excess of the LD_{50}. The greater toxicity may be a reflection of other uncharacterised components present in the crude ricin preparation used in this study. The ricin dose administered by the oral route was of greater lethality than had been intended and, for this reason, the study time period had to be limited to no more than 24 hours. Ricin 'equivalent' protein was, however, detectable in blood, although quantitatively the least amount of toxin found of all tissues investigated. This is almost certainly a reflection of both the small release rate of ricin from the gut but also that it becomes rapidly absorbed by the peripheral tissues as blood circulates. In order for clinical diagnosis to be made blood sampling is the most realistic option despite the relatively low concentration of toxin. This low level detection of ricin will, however, serve purpose for identification of exposure. Unfortunately, exposure to some low (sub-lethal) doses of ricin may not be detectable using this system due to the limited recovery from certain tissues.

Earlier studies by various workers have traced the fate of orally dosed ricin from the intestine to locations in the spleen and liver (Ramsden et al., 1989; Fodstad et al., 1976; Ishiguro et al., 1992b; Ishiguro et al., 1992a). By far the greatest amount of the ricin 'equivalent' was measured in the liver; up to three fold that of the spleen. It is known that the Kupffer cells (reticuloendothelial, scavenging like cells) of the liver absorb ricin from the circulating blood by more than one receptor-ligand type of interaction involving ricin lectin to liver galactose glycoproteins or lipids but also ricin-associated-mannose sugars with liver cell lectins (Bingen et al., 1987; Magnusson et al., 1991; Magnusson et al., 1993; Brech et al., 1993; Frénoy et al., 1992).

The spleen was next in order in terms of the comparative concentration of ricin 'equivalent'. Once antigen-presenting cells, including macrophages, have taken up antigen they migrate to the spleen and other secondary lymphoid tissues, in order to communicate closely with immune competent cells such as T lymphocytes. Hence, the spleen may be exposed to ricin by this mechanism but also, since this organ is richly perfused by blood, it would be exposed to any blood-borne ricin. This was also noted following intramuscular dosing with ricin (Leek et al., 1990).

A range of assay methods for detection of known ricin samples in solution have been described including hydrogel-based protein microchips (Rubina et al. 2005), sandwich type ELISA (Shyu, H. F et al., 2002), chemiluminescence (Poli et al., 1994) or immobilised capture system with silver amplified colloidal gold reporter reagent (Shyu, R. H et al., 2002). A magnetoelastic sensor platform, combined with enzyme immunoassay has been used to determine ricin concentrations in aqueous media (Grimes et al., 2005) but this assay was approximately 25 fold less sensitive than that reported here. Finally, a generic approach to identifying the action of ribosome inhibiting proteins through quantitation of liberated adenine was described by Heisler *et al* (Heisler et al., 2002). This approach would provide evidence of exposure to one of these toxins, which might include ricin but could also be abrin, modeccin, viscumin, volkensin or others. None of the methods were applied to the quantitation of ricin extracted from body tissue samples and the specific assays were of similar or lower sensitivity to the assay described in this paper.

Since the assay antibodies recognise toxin, agglutinin and possibly their breakdown products, this could over-estimate the "toxin" content, which could prove beneficial for the detection of ricin poisoning. However, the assay was fit for purpose and these factors do not affect the ultimate aim of this study as any ricin 'equivalent' detected will be indicative of exposure to the toxin. Now that this method has been confirmed as a sensitive assay for determination of ricin exposure from tissue sampling, further work could be carried out to characterise and optimise the method.

In summary, the ELISA used in this study was sufficiently sensitive to demonstrate measurable quantities of ricin 'equivalent' released from tissues sampled following exposure to the crude toxin. Detection was successful up to 48 hours after exposure and the sensitivity of the assay is great enough to determine levels of ricin above 200pg/ml sample. Thus the assay is likely to be capable to detect ricin exposure which may be encountered in terrorist type scenarios. The method therefore offers promise in terms of diagnosis for the timely implementation of therapy, providing that samples were available soon after the suspected exposure, or for medico-legal purposes.

References

Aplin, P.J., Eliseo, T. (1998) Ingestion of castor oil plant seeds (comment). Comments in Med. J. Aust. 168, 423-424.

Barbieri, L. Batelli, M.G., Stirpe, F. (1993) Ribosome-inactivating proteins from plants. Biochim. Et. Biophys. Acta. 1154, 237-282.

Bingen, A., Creppy, E.E., Gut, J.P., Dirheimer, G., Kirn, A. (1987) The Kupffer cell is the first target in ricin-induced hepatitis. J. Submic. Cyto. 19, 247-256.

Bradberry, S.M., Dickers, K.J., Rice, P., Griffiths, G.D., Vale, J.A. (2003) Ricin poisoning. Toxicol. Rev. 22, 65-70.

Brech, A., Magnusson, S., Stang, E., Berg, T., Roos, N. (1993) Receptor-mediated endocytosis of ricin in rat liver endothelial cells. An immunocytochemical study. Eur. J. Cell Biol. 60, 154-162.

Brown, R.F.R., White, D.E. (1997) Ultrastructure of rat lung following inhalation of ricin aerosol. Int. J. Exp. Path. 78, 267-276.

Dobbs, L. (1989) Markers for differentiated alveolar cells. In: D.G Thomassen and P. Nettesheim. (Eds), Toxicology and carcinogenesis of respiratory epithelium. Hemisphere Publishing Corporation, Taylor Francis Group, Pp 67-75.

Doebler, J.A., Wiltshire, N.D., Mayer, T.A., Estep, J.E., Moeller, R.B., Traub, R.K., Broomfield, C.A., Calamaio, C.A., Thompson, W.L., Pitt, M.L. (1995) The distribution of [^{125}I]ricin in mice following aerosol inhalation exposure. Toxicol. 98, 137-149.

Fodstad, O., Olsnes, S., Pihl, A. (1976) Toxicity, distribution and elimination of the cancerostatic lectins abrin and ricin after parenteral injection into mice. Br. J. Cancer 34, 418-425.

Frénoy, J-P., Turpin, E., Janicott, M., Gehin-Fouque, F., Desbuquois, B. (1992) Uptake of injected 125I-ricin by rat liver *in vivo*. Biochem. J. 284, 249-257.

G D Griffiths, C D Lindsay, A C Allenby, S C Bailey, J W Scawin, P Rice and D G Upshall. (1995) Protection against inhalation toxicity of ricin and abrin by immunisation. Human & Experimental Toxicology, 14, 155-164.

Gareth D Griffiths, Stephen C Bailey, Joy L Hambrook, Martin Keyte, Promukh Jayasekera, John Miles and E D Williamson. (1997) Liposomally-encapsulated ricin toxoid vaccine delivered intratracheally elicits a good immune response and protects against a lethal pulmonary dose of ricin.. Vaccine, 15:7, 1933-1939.

Gareth D Griffiths, Stephen C Bailey, Joy L Hambrook and Martin P Keyte. (1998) Local and systemic responses against ricin toxin promoted by toxoid or peptide vaccines alone or in liposomal formulations.. Vaccine, 16:5, 530 –535.

Gill, D.M. (1982) Bacterial toxins: a table of lethal amounts. Microbiol. Rev. 46, 86.

Griffiths, G.D., Rice, P., Allenby, A.C., Bailey, S.C., Upshall, D.G. (1995) Inhalation Toxicology and Histopathology of Ricin and Abrin Toxins. Inhalation Tox. 7, 269-288.

Grimes, C.A., Ruan, C., Zeng, K., Shankar, K. (2005) Quantification of ricin concentrations in aqueous media. Sens. Actuator B-Chem. 107, 640-648.

Heisler, I., Keller, J., Tauber, R., Sutherland, M., Fuchs, H. (2002) A colorimetric assay for the quantitation of free adenine applied to determine the enzymatic activity of ribosome-inactivating proteins. Anal. Biochem. 302, 114-122.

Ishiguro, M., Nakashima, H., Tanabe, S., Sakakibara, R. (1992a) Interaction of toxic lectin ricin with epithelial cells of rat small intestine *in vitro*. Chem. Pharm. Bull. 40, 441-445.

Ishiguro, M., Tanabe, S., Matori, Y., Sakakibara, R. (1992b) Biochemical studies on oral toxicity of ricin.IV. A fate of orally administered ricin in rats. J. Pharmacobio-Dyn. 15, 147-157.

Jiminez, A., Vasquez, D. (1985) Plant and fungal protein and glycoprotein toxins inhibiting eukaryotic protein synthesis. Ann. Rev. Microbiol. 39, 649-672.

Kopferschmitt, J., Flesch, F., Lugnier, A., Sauder, P., Jaeger, A., Mantz, J.M. (1983) Acute voluntary intoxication by ricin. Hum. Toxicol. 2, 239-242.

Leek, M.D., Griffiths, G.D., Green, M.A. (1990) Pathological aspects of ricin toxicity in mammalian lymph node and spleen. Med. Sci. & the Law. 30, 141-148.

Leith, A.G., Griffiths, G.D., Green, M.D. (1988) Quantification of ricin toxin using a highly sensitive avidin/biotin enzyme-linked immunosorbent assay. J Forensic Science Soc. 28, 227-236.

Lord, J.M., Roberts, L.M., Robertus, J.D. (1994) Ricin: structure, mode of action and some current applications. FASEB J. 8, 201-208.

Magnusson, S., Berg, T., Turpin, E., Frénoy, J-P. (1991) Interactions of ricin with sinusoidal endothelial rat liver cells. Biochem. J. 277, 855-861.

Magnusson, S., Kjeken, R., Berg, T. (1993) Characterization of two distinct pathways of endocytosis of ricin by rat liver endothelial cells. Exp. Cell Res. 205, 118-125.

Olsnes, S., Pihl, A. (1982) Toxic lectins and related proteins. In: P. Cohen and S. Van Heyningen (Eds), Molecular Action of Toxins and Viruses, Elsevier Biomedical Press, pp. 51-105.

Poli, M.A., Rivera, V.R., Hewetson, J.F., Merrill, G.A. (1994) Detection of ricin by colorimetric and chemiluminescence ELISA. Toxicon. 32, 1371-1377.

Ramsden, C.S., Drayson, M.T., Bell, E.B. (1989) The toxicity, distribution and excretion of ricin holotoxin in rats. Toxicol. 55, 161-171.

Rubina, A.Y., Dyukova, V.I., Dementieva, E.I., Stomakhin, A.A., Nesmeyanov, V.A., Grishin, E.V., Zasedatelev, A.S. (2005) Quantitative immunoassay of biotoxins on hydrogel-based protein microchips. Anal. Biochem. 340, 317-329.

Shyu, H.F., Chiao, D.J., Liu, H.W., Tang, S.S. (2002) Monoclonal antibody-based enzyme immunoassay for detection of ricin. Hybrid Hybrido. 21, 69-73.

Shyu, R.H., Shyu, H.F., Liu, H.W., Tang, S.S. (2002) Colloidal gold-based immunochromatographic assay for detection of ricin. Toxicon. 40, 255-258.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. Analyt. Biochem. 150, 76-85.

Stirpe, F., Barbieri, L., Batelli, M.G., Soria, M., Lappi, D.A. (1992) Ribosome-inactivating proteins from plants: present status and future prospects. Biotechnology. 10, 405-412.

Taatjes, D.J., Barcomb, L., Leslie, K.O., Low, R.B. (1990) Lectin-binding profiles of rat lung alveolar epithelial cells. Acta Histochem. Suppl. 40,109-112.

Vogel, P., Rivera, V.R., Pitt, M.L., Poli, M.A. (1996) Comparison of the pulmonary distribution and efficacy of antibodies given to mice by intratracheal instillation or aerosol inhalation. Lab. Animal Sci. 46, 516-523.

Wannemacher, R. W. and Anderson, J.B. 2006. Inhalation Ricin: Aerosol procedures, toxicology and therapy. *Inhalation Toxicology, Second Edition,* Salem, H. and Katz, S., Eds., CRC Taylor & Francis, Taylor & Francis Group, Bocca Raton, London, New York, Ch. 39: 973-980.

FIGURE LEGENDS

Figure 1 – ELISA ricin standard curve.

Standard curve for ricin resuspended in tissue elution buffer. Purified ricin was diluted to give a range of concentrations: 200, 250, 500, 750, 1000, 1500 and 2000 $pg.mL^{-1}$ in elution buffer and assayed as described above using the amplified sandwich ELISA. In practice, the calibration standards were run on every assay plate in quadruplicate, concurrently with tissue controls, vehicle controls and samples. Points are means of replicate determinations of n=7, ± standard error of the mean, collated from seven different experiments. The ricin content of tissue samples was derived from the standard curve and corrected using the corresponding vehicle control samples. It is referred to as ricin 'equivalent' because the assay will identify *Ricinus* agglutinin in addition to ricin toxin and possible breakdown products.

Tables & Figures

Tables 1a, 1b and 1c – Quantitative analysis of ricin 'equivalent' extracted from selected rat tissue samples

Table 1a – Pulmonary Dose of 3.2µg.kg^{-1} crude ricin

Pulmonary 24hours (n = 6)	[ng/ml][a] ± SEM	Sample Volume (ml)	Total Ricin 'equivalent' (ng)	Ricin 'equivalent' (ng/g tissue)	% Yield of dose
Lung	7.13 ± 0.54	1.61	11.45	7.13	1.43
Lung wash	40.75 ± 2.43	1	40.75	n/a	5.09
L wash cells	3.88 ± 0.1	1	3.87	n/a	0.48

Table 1b - Pulmonary Dose of 3.2µg.kg^{-1} crude ricin

Pulmonary 48hours (n=5)	[ng/ml][a] ± SEM	Sample Volume (ml)	Total Ricin 'equivalent' (ng)	Ricin 'equivalent' (ng/g tissue)	% Yield of dose
Lung	11.38 ± 0.86	3.6	40.89	11.38	5.1
Lung wash	1.25 ± 0.07	1	1.25	n/a	0.16
L wash cells	NOT DETECTED				

Note: ricin is referred to as ricin 'equivalent' because the assay will identify *Ricinus* agglutinin in addition to ricin toxin and possible breakdown products

Table 1c – Oral Dose with 8mg.kg^{-1} crude ricin

Oral 24 hours (n = 6) except where shown	**[ng/ml][a] ± SEM**	**Sample Volume (ml)**	**Total Ricin 'equivalent' (ng)**	**Ricin 'equivalent' (ng/g tissue)**	**% Yield of dose**
Blood (cells) (n = 5)	1.41 ± 0.33	1	1.41	n/a	0.7x10^{-4}
Liver	9.50 ±1.57	5.68	53.96	9.5	26.9x10^{-4}
Gut	21.15 ± 3.74	1.78	6.65 (NS)[b]	31.73 (NS)	3.3x10^{-4} (NS)
Spleen	26.30 ± 4.44	3.90	17.3	39.45	8.6x10^{-4}
Kidney	7.25 ± 2.19	3.25	7.11 (NS)	10.88 (NS)	3.5x10^{-4} (NS)

Note: ricin referred to as ricin 'equivalent' because the assay will identify Ricinus agglutinin in addition to ricin toxin and possible breakdown products

[a] Concentration corrected for dilution

[b] NS denotes ricin detected in samples where the signal was not significantly different from the corresponding vehicle assay.

Figure 1 – ELISA ricin standard curve.