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Protection against inhalation toxicity of ricin and abrin by immunisation

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1 Abrin and ricin are highly toxic plant proteins which are very similar in structure and function and inhibit protein synthesis in eukaryotes.

2 Rats have been immunised against either toxin using formaldehyde-toxoids by three subcutaneous injections at intervals of 3 weeks. For abrin, serum titres in 14 out of 15 rats were raised to between 1 : 12800 and 1 : 51200 after two injections, 6 weeks from the start of the experiment. Titres of between 1 : 256 and 1 : 1024 were also measured in lung washes after challenge with active abrin toxin.

3 The three major antibody classes, IgG, IgM and IgA were present in the immune sera but IgG and IgA only were detected in lung washes. The proportion of IgA to IgG was higher in the lung fluid than in sera. Rats immunised by abrin toxoid were protected against 5 LCt_{50}'s of abrin by inhalation but others exposed to ricin were not.

4 For ricin, serum titres ranged from 1 : 800 to 1 : 25600 after two injections and after a third injection the titre range was the same but population samples were weighted towards the higher titres. All rats immunised with ricin toxoid survived the challenge of 5 LCt_{50}'s of ricin toxin by inhalation over the observation period of 28 days post-challenge.

5 Representative immunised rats (abrin toxoid) were taken at various times post-exposure, humanely killed and tissues were examined for pathological changes. It was concluded that an apparently severe lung lesion occurred at a later time than in non-immunised, toxin challenged rats. This damage was not lethal over the experimental observation periods.

6 Immunisation by the sub-cutaneous route therefore protects against lethality from challenge by inhalation of ricin or abrin toxins but does not prevent significant lung damage.

Keywords: ricin; abrin; inhalation; vaccination; protection

Introduction

Abrin and ricin are highly toxic plant proteins which inhibit protein synthesis by preventing the binding of elongation factor 2 to the ribosome. They are therefore described as ribosome-inactivating proteins.[1] When administered systemically in mice, abrin is more toxic than ricin (LD_{50}'s[2] : abrin 0.7 μg kg^{-1}, ricin 2.7 μg kg^{-1}) by a factor of approximately four fold, whereas by inhalation, the potency of these toxins was found to be very similar.[3] Supralethal doses of the toxins do not cause rapid onset of symptoms of intoxication,[4] irrespective of the route of administration. Indeed, lethal effects are not seen following inhalation of very high doses of either toxin until at least 18–24 h after exposure.[3]

The toxicities of ricin and abrin to bovine pulmonary endothelial cells have been compared by measuring the concentration of toxin which reduces the viability of these cells by 50% (the LC_{50}). The LC_{50}'s of abrin and ricin were very similar to systemic *in vivo* toxicities, with abrin being between three to six fold more toxic than ricin, depending on the method used to assess viability.[5] The decrease in cellular viability did not occur until between 15 and 30 h after incubation with lethal concentrations of ricin or abrin. Inhibition of protein synthesis, however, can be detected after exposing to the same concentrations of these toxins for between 30 and 90 min. Biochemical changes, therefore, clearly occur early after contact with ricin or abrin while death occurs much later. If these *in vitro* findings are parallelled *in vivo* then the time for therapeutic intervention after exposure will be very short but immunisation may prevent lethality. Some cross-reactivity between antisera raised to abrin and ricin has been reported[4] and it would be of value to investigate whether immunisation with abrin toxoid offered any protection against challenge with ricin toxin and vice-versa. This study is the first examination of the use of a formaldehyde toxoid of abrin as an immunogen to protect rats from a supralethal inhalation challenge to native toxin aerosol.

We were interested to determine if a simple immunisation scheme using a formaldehyde toxoid would protect against a supralethal inhalation challenge. We also wished to determine the number of toxoid doses necessary to give protective immunity.

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Methods

Purification of toxins
Seeds of *Ricinus communis* (containing ricin) were purchased from N.L. Chrestensen, Markstrasse 38, Erfurt, Germany through a British supplier (Anthony Andrews, Bourne, Leicester). *Abrus precatorius* seeds (containing abrin) were obtained from Sandeman seeds, Sutton, West Sussex.

Both toxins were purified by a method based on those for the isolation of *Viscum album* lectin[6] and ricin toxin.[7] For the preparation of ricin toxin only, castor oil and other seed lipid was removed by solvent extraction in 40–60% petroleum ether[7] from a homogenate of the seeds in 0.5 M NaCl. The seed homogenates were centrifuged to remove large seed debris and the supernatant was fractionated using ammonium sulphate precipitation (60%, w/v), followed by affinity chromatography on acid treated[8] Sepharose 4B, gel filtration on Superdex 2000 and finally on G25 Sephadex. All chromatography was performed using FPLC (Pharmacia). Product purity was assessed using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, the protein bands being visualised by silver staining using a kit (Pharmacia) with a stated detection limit of 0.3–0.5 ng of protein per band. Purity was assessed to be 100% using this method.

Preparation of toxoids
Abrin toxin was treated with 1% (final concentration) formaldehyde in phosphate buffered saline for 2 weeks at 37°C. Unreacted formaldehyde was removed by desalting on G25 Sephadex before the toxoid was tested for toxicity with cells in culture or used for immunisation.

Ricin was treated with formaldehyde at 1% or 10% (final concentrations) for 4 weeks before formaldehyde removal and assay *in vitro* for residual toxicity.

Examination of toxoid in vitro for residual toxicity
The toxoid preparation was assessed for residual toxicity in bovine pulmonary aortic endothelial (BPE) cells grown to confluence in 96 well plates under routine culture methods at 37°C in an atmosphere of 95% O_2, 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum (including routine supplements and antibiotics).

Cells in replicate wells were incubated with three concentrations (10.0, 1.0 and 0.1 μg ml^{-1}) of ricin or abrin toxoid and cells in parallel wells were exposed to the same concentrations of either toxin. Control cells were incubated with growth medium only. After 24 h incubation, toxicity was assessed using the neutral red viability assay.[9,10]

Animals
For the abrin study, 15 male Porton strain rats were obtained immediately post-weaning (3 weeks old) at a body weight of approximately 60 g. In planning the experiment it was anticipated that four injections of toxoid, each separated by 3 weeks, would be required for an optimal immune response. The design of the inhalation exposure apparatus imposed an upper limit on body size and so very young rats were obtained. In the event, only two or three injections of toxoid were needed, with a consequent reduction in the time required for incubation. For subsequent immunisation with ricin toxoid, 16 larger (150–200 g) animals were used.

Immunisation protocols and experimental design
The rats were immunised with toxoid, test bleeds were made to assess immune status and rats were challenged by inhaled toxin aerosol according to the protocol shown in Figure 1.

Exposure to aerosolised toxin
The toxic material was prepared, handled, and the aerosols generated (using a Liu & Lee constant output nebuliser) within modified Class 111 (BS 5726) microbiological containment cabinets.

The solution from which the aerosols were generated consisted of the toxin (at 2.0 mg ml^{-1}) dissolved in PBS containing fluorescein (20 μg ml^{-1}). All material aerosolized (apart from that inhaled by the rats) was captured on a glass fibre filter at the distal end of the exposure line. The aerosol particles consist mainly of NaCl with fluorescein and toxin as minor components, present in the same ratio as the original solution. This enabled the concentration of the toxin in the aerosol (known airflow for known time) to be determined.

A maximum of six rats at a time were exposed, head only, to 5 LCt_{50}'s (see below) of the toxin aerosol. The aerosol concentration was 1.29 μg L^{-1} and exposure times of 16 and 21 min for abrin and ricin respectively. The mass median aerodynamic diameter (MMAD) of the dry particles was calculated to be 0.9 μm (geometric standard deviation, σ_g = 1.6).

The LCt_{50} for ricin is between 4.5 and 5.9 mg min m^{-3} and for abrin 4.5 mg min m^{-3}.[3] Challenge exposures used here to assess protection afforded by immunisation were approximately five times each LCt_{50} value, 26.2 mg min m^{-3} for ricin and 22.7 mg min m^{-3} for abrin (assuming 100% retention in the lung and a mean minute volume of 0.144 L, determined previously in rats of the same sex, strain and weight, these figures could be expressed as approximate doses of 18.5 and 16.6 μg kg^{-1} for ricin and abrin respectively).

In order to determine whether immunisation with one toxoid gave any protection against inhalation challenge with the other, small groups of immunised rats were cross challenged with toxins at the Cts given above.

IMMUNISATION PROTOCOL

First injection
(3 week interval)

Second injection — first test bleeds for assessment of serum titres
(bleed corresponds with injection [1] in Tables 4a & 4b)
(3 week interval)

Third injection — second test bleeds
(bleed corresponds with injection [2] in Tables 4a & 4b)
(3 week interval)

No further injections — *third test bleeds*
(bleed corresponds with injection [3] in Tables 4a & 4b)
(next day)

Exposure to toxin aerosol — determination of protection against lethality

Figure 1 Protocol followed to immunise rats with abrin or ricin toxoid (all injections were subcutaneous and at a dose of 125 μg kg^{-1} body weight)

Bronchio-alveolar lavage fluid preparations (Abrin study only)

Twenty-four hours after challenge with abrin toxin four immunised and three normal control rats were humanely killed by i.p. injection of Euthatal.

Lavage fluids were sampled from each rat by washing ten times with isotonic saline solution. Washings from each animal were pooled. Pooled washings were concentrated ten-fold, samples were assayed for protein[11] and the remainder was stored at –20°C until analysed.

Analysis of serum samples and lavage fluid (Abrin study only): titres and immunoglobulin classes

Titres and immunoglobulin classes in sera and lavage fluids were all determined using enzyme linked immunoabsorbent assay (ELISA) in 96 well microtitre plates. Primary rat anti-toxin antibodies were detected using peroxidase-labelled anti-rat immunoglobulin secondary antibody reagents (Nordic). The presence of rat antibodies was indicated by the intensity of a coloured enzymic (peroxidase) reaction product.

Abrin or ricin toxin was adsorbed on to the wells of the plates by incubation with solutions of either toxin (at 5 μg ml^{-1} in PBS) overnight at 4°C. Sample sera were incubated with the coated plates to select toxin-specific antibodies (immunoglobulins); dilutions were made in PBS containing bovine serum albumin (1% w/v) and Tween 20 (0.5% v/v). Each antibody layer, primary and secondary, was incubated in the plates for 1 h at 37°C after which plates were washed three times with PBS containing 0.05% Tween 20. The bound immunoglobulins were detected using the secondary antibody reagents, anti-rat immunoglobulins labelled with horseradish peroxidase enzyme [HRPO] (Nordic). When incubated with ABTS (azinobis-ethylbenzthiazoline sulphonic acid; Sigma) in 0.15 M citrate/phosphate buffer pH 4.3 containing H_2O_2 (0.03% v/v), a coloured product was formed. The optical density of this solution at 414 nm was proportional to the amount of the rat anti-toxin immunoglobulins bound to either toxin on the plate.

To determine end-point titres, samples of rat sera containing the primary antibodies were serially diluted across the plate before incubation; bound rat immunoglobulins were again detected using HRPO-labelled anti-rat immunoglobulins (Nordic). The end point titre was taken as the serum dilution at which the absorbance (at 414 nm) was at least 0.1 unit when that of the corresponding control dilution had been subtracted.

To detect the immunoglobulin classes of rat antibodies with anti-toxin specificity, toxin coated plates were incubated with rat sera (at 1 : 50 dilution), washed and secondary detection antibodies added. Here, four antibodies labelled with horseradish peroxidase were used (Nordic Laboratories), each having specificity for different rat immunoglobulin classes (IgG, IgA, IgM or IgE).

In the titration or immunoglobulin class determining ELISAs, any non-specific absorbance was eliminated by subtracting the absorbance recorded from the pooled sera of control animals.

In addition to measuring titres of individual rat serum samples, pools of sera for each bleed group within each toxoid study were made and group mean titres measured. This enabled a simple comparison to be made between the immune status for each group after each injection.

Histopathology of immunised rats post-challenge

Three animals, immunised by abrin toxoid and exposed to a challenge of 5 LCt_{50}'s of abrin by inhalation, were examined for histopathology at 1 day following challenge. A similar group was examined after 14 days. Control animals (a separate group which had not been immunised) were also exposed to 5 LCt_{50}'s of abrin. Two of these animals were culled at 1 day and examined. The two animals immunised with abrin toxoid and cross-challenged with 5 LCt_{50}'s of ricin were examined at 2 days post-challenge.

The four rats immunised with ricin toxoid and challenged with 5 LCt_{50}'s of abrin were not examined for histopathology. The remaining 12 immunised rats challenged as above with ricin were observed for clinical signs. These rats were culled at the end of the 28 day observation period and tissues examined for histopathology.

The lungs from the animals of these groups were carefully excised *en bloc* with the trachea and larynx following incision of the chest wall. The tissues were immersion fixed in neutral buffered formalin at room temperature for a period of not less than 48 h. After this time, each lung was trimmed to produce representative blocks from both left and right lobes; sections 5 ± 2 µm thick were cut from paraffin wax embedded material and routinely stained with Harris's haematoxylin and eosin. The resulting histological sections were examined at magnifications up to × 630 on a Zeiss Axioplan photomicroscope.

Observation of rat weights over the entire experimental period and clinical signs post-challenge

Rats were weighed routinely on alternate days during the 9 week immunisation period of each study and every day for the first ten days post-challenge, reverting to alternate days for the remaining period. Observations were also made of the physical appearance of the rats, their activity levels and general signs of malaise.

Results

Assessment of formaldehyde toxoiding

The NR viability assay in BPE cells showed a large difference between the effects of the toxoid (produced by treating abrin with 1% formaldehyde) and equal concentrations of native abrin toxin. In cells treated with toxoid at 10 µg ml^{-1} (the highest concentration of toxoid tested) viability was reduced to 76.1% of control values. Abrin toxin, at this concentration, reduced cell viability to 4.4%. The formaldehyde treatment of abrin toxin had therefore greatly reduced its toxicity and it was considered suitable for use in the immunisation programme.

Ricin treated with 1% formaldehyde for 4 weeks was assayed at 10 µg ml^{-1} with BPE cells. Viability of the cells was reduced to 15.7% of control cells, while an equivalent concentration of ricin toxin reduced viability to 7.0%. After treatment with formaldehyde at 10% for 4 weeks, the highest concentration of toxoid caused no significant reduction in cell viability, whereas the toxin at an equivalent concentration reduced cell viability to 8.0%. This preparation was considered satisfactory for use as a vaccine.

Table 1 Outcome of challenge with abrin or ricin toxins on immunised and non-immunised groups

Challenge	*Immunised with ricin toxoid*		*Immunised with abrin toxoid*		*Non-immunised controls*	
5 LCt_{50} toxin	*A*	*R*	*A*	*R*	*A*	*R*
Number of rats	4	12	6	2	3	3
Mortality	4/4	0/12	0/6	2/2	3/3	3/3
(Observation period, days)	(2)	(28)	(14)	(2.5)	(1)	(3)

A = abrin; R = ricin.
Immunised groups received three subcutaneous injections of toxoid at 3 week intervals.

Table 2 Development of rat serum titres against toxins after repeated toxoid immunisation

a — Abrin toxoid
Titres in sera from bleeds taken at 3 week intervals after each toxoid injection (numbers of rats)

Titre	*50^{-1}*	*100^{-1}*	*200^{-1}*	*400^{-1}*	*800^{-1}*	*1600^{-1}*	*3200^{-1}*	*6400^{-1}*	*12800^{-1}*	*25600^{-1}*	*51200^{-1}*	*102400^{-1}*
Inj.[1]	–	–	(1)	–	(7)	(5)	(2)	–	–	–	–	–
Inj.[2]	–	–	–	–	–	–	–	(1)	(4)	(5)	(4)	(1)
Inj.[3]	–	–	–	–	–	–	–	–	(2)	(6)	(6)	(1)

b — Ricin toxoid
Titres in sera from bleeds taken at 3 week intervals after each toxoid injection (numbers of rats)

Titre	*50^{-1}*	*100^{-1}*	*200^{-1}*	*400^{-1}*	*800^{-1}*	*1600^{-1}*	*3200^{-1}*	*6400^{-1}*	*12800^{-1}*	*25600^{-1}*	*51200^{-1}*	*102400^{-1}*
Inj.[1]	(3)	(5)	(2)	(3)	(3)	–	–	–	–	–	–	–
Inj.[2]	–	–	–	–	(2)	(4)	(3)	(3)	(2)	(1)	–	–
Inj.[3]	–	–	–	–	(1)	(1)	(4)	(2)	(6)	(2)	–	–

Rats received three subcutaneous toxoid injections of 125 µg kg^{-1} at 3 week intervals.
The titre of the serum samples is that dilution of serum whose absorbance at 414 nm (ABTS immunoperoxidase assay) is ≥0.1 absorbance units above that of the corresponding dilution of non-immune (control) serum.

Table 3 Distribution of anti-toxin specificity between immunoglobulin classes in sequential serum pools

a — Pools of serum samples from 15 rats injected with abrin toxoid, covering response to three injections over 9 weeks. Titres of pooled serum samples are also shown.

Serum pool relating to toxoid injection (weeks)	*Percentage distribution of immunoglobulin classes*			
	IgG	*IgA*	*IgM*	*Pooled serum titre*
Injection [1] (3)	63.2	13.0	23.8	3200^{-1}
Injection [2] (6)	80.7	12.9	6.4	51200^{-1}
Injection [3] (9)	77.8	12.8	9.4	102400^{-1}

b — Pools of serum samples from 16 rats injected with ricin toxoid, covering response to three injections over 9 weeks. Titres of pooled serum samples are also shown.

Serum pool relating to toxoid injection (weeks)	*Percentage distribution of immunoglobulin classes*			
	IgG	*IgA*	*IgM*	*Pooled serum titre*
Injection [1] (3)	55.2	11.7	33.1	200^{-1}
Injection [2] (6)	64.4	12.7	**22.9**	3200^{-1}
Injection [3] (9)	79.2	11.9	8.9	12800^{-1}

Immunisations were performed with abrin toxoided for 2 weeks with 1% formaldehyde and ricin toxoided for 4 weeks with 10% formaldehyde after excess formaldehyde was removed by gel chromatography.

Effect of immunisation on lethal challenge
Rats were immunised by the protocol shown in Figure 1. Both toxoid treatment groups were protected from lethal challenge with the corresponding toxin by inhalation whereas non-immunised rats died (Table 1). However, immunisation with abrin toxoid failed to protect rats against challenge with 5 LCt_{50}'s of aerosolised ricin. Similarly, immunisation with ricin toxoid failed to protect against challenge with abrin toxin.

Development of humoral immunity

(A) Abrin: Three weeks after the second toxoid injection, titrations of serum samples showed end points at high dilution (Table 2a). These ranged from 6400^{-1} to 102400^{-1} with five out of fifteen rats sera titrating to 25600^{-1}. A third toxoid injection produced two rats out of fifteen with serum titres of 12800^{-1}, six with serum titres of 25600^{-1} and six titrating to 51200^{-1} (1 at 102400^{-1}). Pooled samples of each set of sera (corresponding with injections 1–3) were later performed and results are shown in Table 3a. The third toxoid injection increased the group mean titre by one serial dilution from 51200^{-1} to 102400^{-1}.

(B) Ricin: For direct comparison with the abrin data, three weeks after the second toxoid injection (Table 2b) serum titres ranged from 800^{-1} to 25600^{-1} with nine samples at or below 3200^{-1} and six at or above 6400^{-1}. A third injection of toxoid failed to increase the highest titre of the range but produced ten sera with titres of 6400^{-1} or above (six at 12800^{-1}). The result of titrations of pooled serum samples corresponding with each injection are shown in Table 3b. Here, the second injection produced a group mean serum titre of 3200^{-1} and the third of 12800^{-1}, an increase of two serial dilutions.

Immunoglobulin classes in pooled samples of sera having anti-toxin specificity.

(A) Abrin: Three weeks after the first injection of toxoid 63.2% of the specificity was confined to the IgG class, 23.8% to the IgM class and 13.0% was IgA (Table 3a). IgE was found in only one rat and is not recorded in the table. After the second injection of toxoid, anti-abrin activity was switched mainly from IgM to IgG class. The distribution ratios were now approximately 81 : 13 : 6 (IgG : A : M respectively). There was no general change in this distribution following the third injection of toxoid.

(B) Ricin: Here, only IgG, M and A classes were examined in the pooled serum samples. Three weeks after the first toxoid injection 55.2% of the specificity was in the IgG class, 33.1% IgM and 11.7 IgA (Table 3b). After the second toxoid injection IgG levels had increased to 64.4% at the expense of IgM (22.9%) with IgA changing very little (12.7%). After the third injection of toxoid, IgG levels of anti-ricin were 79.2% while IgM had decreased to 8.9% and IgA had remained almost steady at 11.9%.

Analysis of bronchio-alveolar lung fluid samples (abrin study only)
The end points of the titres were between 256^{-1} and 1024^{-1} and all samples had elevated protein concentrations compared with control levels, 3 to 4-fold in

Table 4 Immunoglobulin classes detected in lung lavage fluids from rats immunised with abrin toxoid and challenged with $5LCT_{50}$'s abrin

Rat code	*Lavage fluid titre* 256^{-1}	*Lavage fluid titre* 1024^{-1}	*Lavage fluid protein (mg ml*$^{-1}$*)*	*Mean % Ig* *IgG*	*Mean % Ig* *IgA*	*Serum titre Inj. [3]*
Control	–	–	0.70 (mean, n=3)	–	–	–
A		X	2.38			51200^{-1}
V	X		1.52	66.2 ± 40.5	33.8 ± 16.9	51200^{-1}
C		X	2.10			51200^{-1}
D	X		1.72	(n=4)	(n=4)	51200^{-1}

Lavage fluid taken 24 hours post-challenge
X = titre measured.
Lung lavages from each animal consisted of ten, 5 ml washes pooled and concentrated ten-fold. Protein contents were determined.[11]
The pools were analysed specifically for anti-abrin antibodies using ELISA.
Samples were assayed also for the presence of IgM and IgE classes which were found to be absent.

some cases (Table 4). The mean protein concentration (mg ml^{-1}) of lavage fluids from immunised rats challenged with abrin toxin aerosol was 1.93 compared with 0.7 control. Lavage fluids showed very similar immunoglobulin content being approximately 66% IgG and 34% IgA. The percentage of IgA in lavage fluid was, therefore, higher than that in serum.

Histopathology of abrin toxoid immunised rats after inhalation challenge with abrin and ricin toxin

Rats immunised with abrin toxoid and challenged with abrin appeared clinically normal after 24 h, although histologically there was evidence of mild focal interstitial oedema of the lung but no haemorrhage (Figure 2). At 14 days the animals showed severe pulmonary pathology indicating that immunisation had only delayed the onset of significant lung damage. (Immunised animals had died 48 h after being challenged with ricin). Histologically the lungs showed generalised interstitial oedema and moderate intra-alveolar/intra-bronchiolar haemorrhage (Figure 3). The interstitial tissues were inflamed with large numbers of macrophages and small lymphocytes. Occasional necrotic neutrophils were also noted and the presence of apoptotic bodies was minimal. The larger airway epithelium was intact and non-inflamed and a few animals showed severe but focal type II cell hyperplasia. In summary, the pathology reported in the immunised rats at 14 days post-challenge with abrin aerosol (5 LCts) was similar to that of non-immunised rats at 3 days post-exposure to abrin aerosol.

A total of three animals were available for examination following challenge with ricin holotoxin and all had died 48 h after challenge. Before death, the animals appeared to have been clinically unwell with significant pilorection, central cyanosis and hyperinflation of the chest. Histologically, the lungs

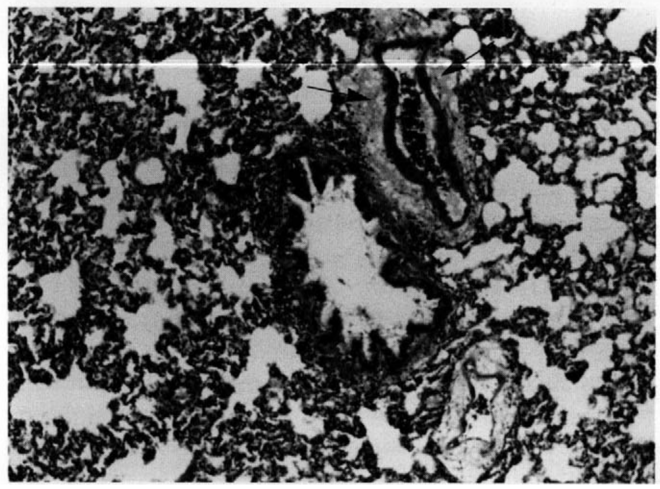

Figure 2 Section of lung tissue from an animal immunised with abrin toxoid and examined one day post-challenge with 5 LCt_{50} of abrin. The lung shows significant interstitial, predominantly perivascular, oedema (arrows). Haematoxylin and eosin 3325

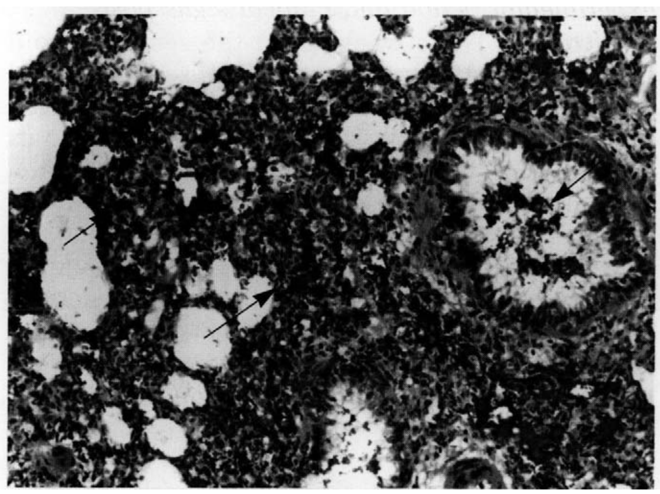

Figure 3 Section of lung tissue from an animal immunised with abrin toxoid and examined fourteen days post-challenge with 5 LCt_{50} of abrin. The lung shows significant intra-alveolar and intrabronchiolar haemorrhage (arrows). Haematoxylin and eosin 3325

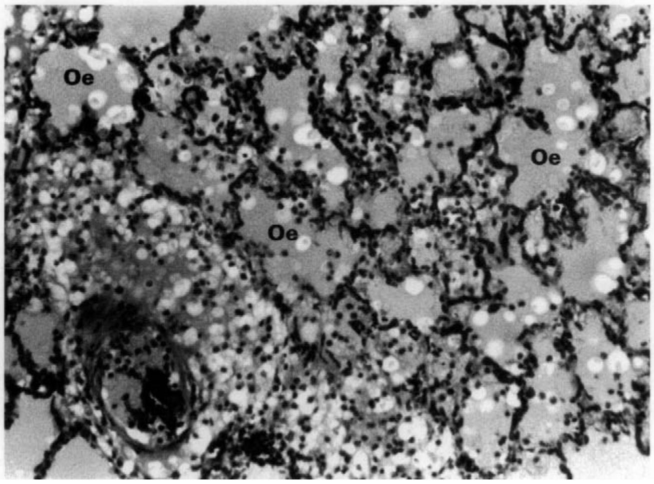

Figure 4 Section of lung tissue from an animal immunised with abrin toxoid and examined two days post-challenge with 5 LCt_{50} of ricin. The lung shows significant intra-alveolar oedema (Oe), acute alveolitis and alveolar capillary congestion. Haematoxylin and eosin 3325

showed severe, overwhelming intra-alveolar oedema accompanied by established acute, destructive alveolitis with significant apoptosis at the alveolar surface (Figure 4). The epithelium lining the larger airways was frankly necrotic and the liver, spleen and kidneys all showed severe passive venous congestion with hepatic sinusoidal ectasia.

Histopathology of ricin toxoid immunised rats after inhalation challenge with ricin toxin
Rats which were immunised with ricin toxoid and challenged with ricin aerosol as above survived through the 28 day observation period post-exposure when they were humanely killed. Histologically, their lungs appeared normal.

Observation of rat weights over the entire experimental period and clinical signs post-challenge
Both ricin and abrin groups of rats maintained a fairly steady weight gain throughout the immunisation periods until challenge with respective toxins. A close examination of the body weight data curves (not shown) showed small plateaus in each which corresponded with time periods shortly after injections of toxoided proteins. These were more marked for ricin than for abrin toxoid. After challenge with toxin aerosols, the abrin toxoid group exhibited a maximal weight loss of 2.5% bodyweight at 2 days post-exposure while the ricin toxoid group experienced a maximum weight loss of 5.2% at 3 days. The percentages were calculated from the group mean minimum bodyweight compared with the group mean weight on the day before challenge. In comparison, unprotected rats, when challenged lost up to approximately 30% of bodyweight before death.

Observations of animal activity and general well-being showed no adverse signs in the abrin toxoid group post-challenge, over the 14 day observation period. The ricin toxoid group were unwell by 2 days post challenge being inactive with laboured respirations. Half of the group lost weight, three exhibited piloerection and one was immobile with half-closed eyes. Some improvement was evident by 3 days although half of the group were still losing weight; respirations were less laboured for ten of the group, piloerection was reduced and only five rats were persistently immobile. At 6 days, all rats had gained weight, seven weighing more than immediately before the challenge and only two rats now showed laboured respirations. Four animals remained relatively inactive but piloerection was now not apparent. By 7 days all rats had recovered, since no apparently abnormal signs could be seen, and now began gaining weight throughout the remainder of the 28 day observation period.

The non-immunised rats and also the immunised but cross-challenged groups showed signs of intoxication from between 19 and 24 h post-exposure. These signs included reduced activity, closed eyes, hunched posture, piloerection and laboured respirations. All animals lost weight and died between 27 and 69 h, by which time discoloured fluid or froth was apparent around the mouth or nose. On autopsy, the lungs were grossly congested and colourless fluid exuded when the trachea was cut. The liver, kidneys and spleen were dark coloured.

Discussion

Immunisation with ricin or abrin toxoids by the subcutaneous route has been shown to protect against death from inhalation of lethal amounts (5 LCt_{50}'s) of the corresponding toxin aerosol. Examination of the data showing development of humoral immunoglobulin classes at different stages of the immunisation protocol revealed that high levels of toxin-specific antibodies were achieved after two injections of abrin toxoid. This was especially marked for the abrin toxoid group of rats, which responded better than the ricin toxoid group. The high titres found in serum samples after the second injection of abrin toxoid were only marginally improved by a third while IgA levels remained constant. It is likely that the abrin group of experimental animals were already hyperimmunised after two injections of 125 μg kg^{-1} and that this number of toxoid doses may suffice to produce protection from lethality, especially where the immunogenicity of the antigen is not excessively compromised. Titres were much lower for the ricin toxoid group and this is attributed to the higher concentration of formaldehyde used to toxoid the ricin. It is currently understood that humoral immunity in the mucosal immune system results from secretory IgA as the major immunoglobulin[12] and indeed our results show increased levels of IgA in lung washes, although IgG is the major class detected.

Although immunisation protected against fatalities from inhaled toxin, significant lung pathology clearly developed and the survivors of a toxin aerosol challenge would probably be physically compromised. Sub-lethal lung pathology has been described[13] in mice immunised against ricin, on exposure to the aerosolised toxin. In our study no lung damage was observed at the end of the 28 day observation period post-challenge with inhaled ricin. This may well be because histology was not examined at earlier times and the ricin-challenged rats did show signs of illness with pulmonary symptoms at between 2 and 7 days after toxin inhalation although all recovered and survived. The abrin immunised group, on the other hand, showed no physical signs of illness after challenge with aerosolised abrin although histological examination clearly showed the development of apparently severe lung pathology similar to that observed in unprotected rats.

One possible cause for the pulmonary lesions which developed in immunised animals could be toxin overwhelming the immune response. Toxin which had not been neutralised by antibody, although sub-lethal in amount, may be sufficient to cause tissue damage. If this was so, it might be possible to prevent the damage by, for example, passive immunisation with pre-formed anti-toxin antibodies into the lungs. In order to reduce chances of anaphylactic shock on repeated exposure, the Fab fragments of the antibodies could be used. This preparation retains the toxin-binding property while removing the heavily glycosylated Fc region of the antibody molecule. On the other hand, one may conjecture that the loss of the Fc part of the antibody molecule may compromise uptake of antibody-antigen complexes by the scavenging lung macrophages.[14] Another possible reason for the lung damage might be through release of mediators.

Our histological observations following inhalation of immunised and unprotected rats with ricin or abrin show a marked inflammatory response with pulmonary oedema. During the process of inflammation, a number of cytokines are produced in response to the stimulatory agent.[15] In the septic adult respiratory distress syndrome (ARDS), major features of pathogenesis are adherence of polymorphonuclear neutrophils (PMN) to pulmonary endothelial cells and pulmonary oedema. A growing body of evidence suggests that the cytokine tumour necrosis factor alpha (TNF-α) or cachectin, plays a key role in the mechanism of ARDS.[16] Indeed, infusion of recombinant TNF-α into guinea pigs reproduced the appearance of endotoxin-induced acute lung injury.[17] TNF-α primes endothelial cell monolayers to potentiate PMN adhesion and, upon activation of PMN, leads to increased monolayer permeability.[18] Evidence suggests that TNF-α may also directly increase pulmonary vascular permeability[19] and it has been shown to be cytotoxic to primary cultures of bovine aortic endothelial cells, leading to massive deletion by apoptosis.[20] Recently, Stirpe *et al.*[21] have shown that ricin induces the release of TNF-α and interleukin-1β by human peripheral-blood mononuclear cells and detected levels of TNF-α in plasma taken from rats injected with ricin. The involvement of this mediator in the development of the ricin and abrin lung lesions is currently under investigation.

The immunogenicity of the two toxoids used for immunisation might also contribute to differences in the performance of the immunisation treatment upon aerosol challenge with toxin. Abrin was toxoided in the presence of 1% formaldehyde while ricin was apparently unaffected in 4 weeks. Ricin was, however, rendered non-toxic in cell culture after treatment with 10% formaldehyde for the same time period. One may speculate that although this treatment was necessary to make the toxoid enzymically inactive, it is possible that epitopes would be masked by excessive cross-linking compared with the abrin toxoid. The ricin toxoid would therefore be a poorer immunogen against challenge by ricin toxin than the abrin toxoid would be against abrin. Further studies are underway to produce a better ricin toxoid, although the material used in this work did protect against death from exposure to potentially lethal amounts of ricin aerosol, early clinical signs of illness were noted on ricin challenge but were absent in the abrin study group. We are also currently investigating alternative delivery systems for immunisation. It has been reported that exposure of immunised rabbits to sub-lethal dosage of airborne ricin produced a marked boost to titre in contrast to the effect of boosting by injection.[22] Therefore, it may also be worthwhile examining other routes of immunisation.

Alternative strategies of protection against the toxicity of ricin have been examined. Hewetson *et al.*[23] protected mice against inhaled ricin by vaccination with five injections of low doses of native ricin toxin in either Freund's or Ribi™ adjuvants. In our study we investigated the protection given by vaccination with harmless toxoids in the absence of adjuvant and found that three injections of toxoid were sufficient to confer protection against lethality from inhaled toxin challenge. In another study[24] polyclonal BALB/c mouse or rabbit anti-idiotype sera were prepared (against a neutralising goat anti-ricin IgG preparation) and used *per se* or conjugated to keyhole limpet haemocyanin. Injection of the anti-idiotypes from conjugated antibody or the rabbit anti-idiotypes into BALB/c mice elicited specific anti-ricin immune responses but protection against *in vivo* challenge was not performed. Protection against intravenous ricin challenge has also been achieved by administration of monoclonal antibody against ricin.[25] Following challenge, the mice were found to be protected from subsequent challenge at 21 or 35 days later indicating that active immunization had resulted from this two stage procedure. Clearly, any immunisation involving native toxin would have dubious application to human prophylaxis. Passive immunisation by intraperitoneal injection of a monoclonal antibody which neutralised toxin action *in vitro*, delayed time to death from subcutaneous challenges, 18 h later, with a range of ricin doses.[26] The administration of this antibody before and after challenge achieved better protection.

Analysis of the classes of specific anti-abrin immunoglobulins in serum samples showed that both IgG and IgA were present in a ratio of approximately 6 : 1 respectively. Each lung fluid sample had a similar titre and similar ratio of IgG to IgA, and no other classes were detected. Perhaps of more interest is that the ratio of IgA to IgG was higher in lavage fluid (1 : 2) than in serum (1 : 6), reflecting the role of IgA in mucosal immunity.[12] Protein levels were high in the lavage fluid taken from the immunised group. It is not clear whether this is due to the presence of proteinaceous oedema or a high cellular content in the lung washes since samples were not examined microscopically. Certainly, lungs from mice immunised with ricin toxoid[13] had much higher numbers of macrophages in lavage fluids after ricin challenge than non-immunised animals.

In a retrospective examination of immunoglobulin classes present in individual sera sampled from rats immunised with ricin toxoid, several rats had elevated levels of IgE (data not shown). This was particularly true after the first toxoid injection when anti-ricin titres were low. After the second toxoid injection when anti-ricin titres were much higher, the IgE response in those few rats was diminished and completely absent after the third injection. In a separate study (unpublished) we immunised rats with ricin toxoid, challenged with ricin by inhalation and examined serum titres and immunoglobulin classes ten days later. No elevated IgE levels were detected although IgG, IgA and IgM levels were all markedly boosted. Lung fluid immunoglobulins were not investigated. Clearly, Thorpe *et al.*[27] have demonstrated that ricin toxin potentiates IgE responses. Although we were not specifically addressing that phenomenon in our study it would appear that toxoided ricin did not produce elevated levels of anti-ricin IgE in many rats particularly when anti-toxin titres were high. Thorpe *et al.*[27] found that heat denaturation of ricin reduced the IgE potentiation and it is likely that formaldehyde treatment similarly effects the toxin molecule.

In summary, our present study shows that formaldehyde toxoids of ricin and abrin will protect against inhalation challenges with potentially lethal amounts of the respective toxin aerosol although a lung pathology clearly developed with abrin. That no pathology was found in the later ricin experiments may merely reflect experimental design and further investigation is necessary. We are currently examining the role of major cytokines in the pathogenesis of ricin and abrin pulmonary lesions.

References

1 Stirpe F, Barbieri L, Battelli MG, Soria M & Lappi DA. Ribosome-inactivating proteins from plants: present status and future prospects. *Biotechnology* 1992; **10:** 405–12.

2 Gill DM. Bacterial toxins: a table of lethal amounts. *Microbiol Review* March 1982; 86–92.

3 Griffiths GD, Rice P, Allenby AC & Upshall DG. The inhalation toxicology and histopathology of ricin and abrin toxins — Unpublished experimental observations.

4 Olsnes S & Pihl A. Toxic lectins and related proteins. In *Molecular Action of Toxins and Viruses*. Cohen & Van Heyningen eds. Elsevier Biomedical Press, 1982, Ch. 3, pp. 51–105.

5 Lindsay CD, Griffiths GD & Upshall DG. Examination of several protein toxins of plant and bacterial origins using bovine pulmonary endothelial cells. Manuscript in press, *Toxicology* (1994).

6 Ziska P, Franz H & Kindt A. The lectin from *Viscum Album L.* purification by biospecific affinity chromatography. *Experientia* 1978; **34:** 123–4.

7 Cumber AJ, Forrester JA, Foxwell BMJ, Ross WCJ & Thorpe PE. Preparation of antibody-toxin conjugates. *Methods in Enzymology* 1985; **112:** 207–25.

8 Ersson B, Aspberg K & Porath J. The phytohaemagglutinin from sunn hemp seeds (*Crotalaria juncia*). Purification by biospecific affinity chromatography. *Biochimica at Biophysica Acta* 1973; **310:** 446–52.

9 Borenfreund E & Peurner JA. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicology Letters* 1985; **24:** 119–24.

10 Borenfreund E, Babich H & Martin-Alnwick N. Comparisons of two *in vitro* cytotoxicity assays — the neutral red (NR) and tetrazolium MTT tests. *Toxicity In Vitro* 1988; **2:** 1–6.

11 Lowry OH, Rosenbrough J, Farr AL & Randall RJ. Protein measurement with folin phenol reagent. *Journal of Biological Chemistry* 1951; **193:** 265–75.

12 McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M & Kiyono H. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* 1992; **10:** 75–87.

13 Rippy MK, Rivera VR, Hewetson JF, Creasia DA & Zack PM. Immunization with ricin toxoid prevents death and reduces lung injury after aerosol challenge with ricin. Meeting Abstract from: Society of Toxicologic Pathologists, Monterey, 1991.

14 Roitt I. Molecules which recognize antigen. In

Essential Immunology. 7th edition. Blackwell Scientific Publications, 1991, Chapter 3.

15 Van Deuren M, Dofferhoff ASM & Van Der Meer JWM. Review Article, Cytokines and the response to infection. *Journal of Pathology* 1992; **168:** 349–56.

16 Tracey KJ, Lowry SF & Cerami A. Cachectin/TNF-α in septic shock and septic adult respiratory distress syndrome. *American Review of Respiratory Disease* 1988; **138:** 1377–9.

17 Stephens KE, Ishizaka A, Larrick JW & Raffin TA. Tumour necrosis factor causes increased pulmonary permeability and edema. *American Review of Respiratory Disease* 1988; **137:** 1364–70.

18 Gibbs LS, Lai L & Malik AB. Tumour necrosis factor enhances the neutrophil-dependent increase in endothelial permeability. *Journal of Cellular Physiology* 1990; **145:** 496–500.

19 Horvath CJ, Ferro TJ, Jesmok G & Malik AB. Recombinant tumour necrosis factor increases pulmonary vascular permeability independent of neutrophils. *Proceedings of the National Academy of Sciences USA* 1988; **58:** 9219–23.

20 Robaye B, Mosselmans R, Fiers W, Dumont JE & Galand P. Tumour necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells *in vitro*. *American Journal of Pathology* 1991; **138:** 447–53.

21 Licastro F, Morini MC, Bolognesi A & Stirpe F. Ricin induces the production of tumour necrosis factor-α and interleukin-1β by human peripheral-blood mononuclear cells. *Biochemical Journal* 1993; **294:** 517–20.

22 Cope AC, Dec J, Cannan RK & Renshaw B. Ricin. In *Chemical Warfare Agents and Related Chemical Problems*, Volume (1). US Office of Science Research and Development, National Defence Research Committee, Washington DC, 1946, chapter 12, pp. 179–203.

23 Hewetson JF, Rivera VR, Creasia DA, Lemley PV, Rippy MK & Poli MA. Protection of mice from inhaled ricin by vaccination with ricin or by passive treatment with heterologous antibody. *Vaccine* 1993; **11:** 743–6.

24 Chanh TC & Hewetson JF. Polyclonal anti-idiotypes induce antibody responses protective against ricin cytotoxicity. *Immunology* 1993; **79:** 681–4.

25 Lemley PV & Wright DC. Mice are actively immunized after passive monoclonal antibody prophylaxis and ricin toxin challenge. *Immunology* 1992; **76:** 511–13.

26 Chanh TC, Romanowski MJ & Hewetson JF. Monoclonal antibody prophylaxis against the *in vivo* toxicity of ricin in mice. *Immunological Investigations* 1993; **22:** 63–72.

27 Thorpe SC, Murdoch RD & Kemeny DM. The effect of the castor bean toxin, ricin, on rat IgE and IgG responses. *Immunology* 1989; **68:** 307–11.