Biochemical Pharmacology, Vol. 46, No. 2, pp. 327–329. 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

SHORT COMMUNICATIONS

Polyclonal amanitin-specific antibodies: production and cytoprotective properties *in vitro*

(*Received* 15 *March* 1993; *accepted* 13 *April* 1993)

Abstract—The amanitins found in several mushroom species are responsible for many deaths every year. Based on its successful application to cardiac glycoside overdose, immunotherapy could be applicable to amanitin toxicity. Therefore, we produced polyclonal amanitin antibodies by immunizing rabbits with a novel conjugate of α-amanitin. Purified antibodies had an average association constant for α-amanitin of $1.3 \times 10^9\ M^{-1}$. A partially protective effect of the antibodies against amanitin toxicity *in vitro* in Chang cells was evident at a molar ratio of antibody binding sites to α-amanitin of 4:1. Together with reported studies *in vivo*, these investigations indicate the potential of immunotherapy for amanitin poisoning.

Ingestion of *Amanita phalloides* or other mushrooms containing the cyclic octapeptides, the amanitins, accounts for 95% of all fatal cases of mushroom poisoning worldwide [1]. In light of the success in reversing massive digitalis poisoning in humans by digoxin-specific ovine polyclonal Fab fragments [2], and features of the amanitins that make them attractive candidates for immunotherapy, we report the production of polyclonal, amanitin-specific antibodies and their cytoprotective effects in an *in vitro* assay of toxicity in Chang cells.

Methods and Results

The complete synthetic pathway for the preparation of the amanitin conjugates is outlined in Fig. 1 and in Ref. 3. The amanitins were purified from *Amanita phalloides* [4]. To synthesize product I, 1,2-diaminoethane (17.4 mmol) was dissolved in chloroform (20 mL) and cooled to 4°. Di-*tert*-butyl dicarbonate (8.7 mmol) was added dropwise slowly into the stirred solution at 4°. Two hours later, the undissolved material was removed by filtration and the precipitate washed twice with chloroform (2 mL) at 4°. The washings were combined with the original filtrate and evaporated at room temperature. Product I was recrystallized in a 3:2 (v/v) chloroform/*n*-hexane solution to yield needlelike crystals (68.3%, 1.9 g). Nuclear magnetic resonance analysis on a Varian EM 360 spectrometer [deuterated chloroform with 1% (v/v) tetramethylsilane] confirmed the structure (1.4 ppm, 9H; 1.55 ppm, 2H; 3.15 ppm, 4H; 5.05 ppm, 1H). Product II, which was synthesized via the mixed anhydride method, had a melting point of 100–104° consistent with the reported value [5]. Infrared spectral analysis (KBr disc, Perkin Elmer 599 Infrared Spectrometer) also confirmed the structure. To synthesise product III, 10 mg metallic sodium, washed twice in *n*-hexane (3 mL), was dissolved in anhydrous ethanol (10 mL) to produce a sodium ethylate solution. Then, α-amanitin (55 μmol) was dissolved in anhydrous ethanol (2 mL) to which the sodium ethylate solution (55 μmol) was added. The mixture was then evaporated at room temperature. Product IV was obtained by reacting III and 20 mg recrystallized II in 1 mL dried dimethylformamide overnight at room temperature. The mixture was evaporated, dissolved in 20% (v/v) aqueous acetonitrile, filtered and loaded onto a SEP-PAK C_{18} cartridge (Waters, Medford, MA, U.S.A.) that was prewashed with acetonitrile and 5% (v/v) aqueous acetonitrile sequentially. The cartridge was eluted first with acetonitrile and then with 50% (v/v) aqueous acetonitrile to yield 5% product IV. Binding of amanitin antibody to the purified product IV was determined by the nylon net immunoassay [6] using a kit which was a gift from Dr H. Faulstich (Max Planck Institute, Heidelberg, Germany). The wavelength maxima of α-amanitin and IV were identical (302 nm), as was binding to the antibody in the immunoassay. Product V was obtained by the hydrolysis of IV in trifluoroacetic acid and purified using a SEP-PAK C_{18} cartridge. The elution buffers were 10 and 50% (v/v) aqueous acetonitrile, and the yield, 73%. α-amanitin-linker–protein conjugates were prepared by the carbodimide method of Cessi and Fiume [7]. The molar ratio of α-amanitin-linker:protein, determined spectrophotometrically, was 1.7 for bovine serum albumin (BSA*), and 0.8 for ovalbumin.

Amanitin-specific antibodies were elicited in two female adult New Zealand rabbits by immunizing with α-amanitin-linker–BSA. Plasma immunoglobulin G (IgG) was fractionated by protein A chromatography and the amanitin-specific IgG purified by affinity chromatography using an α-amanitin-linker–Sepharose column. This column was prepared from cyanogen bromide-activated Sepharose 4B (2 g). To 2 mL of the prepared resin was added α-amanitin linker (2 mg, V) dissolved in 0.1 M carbonate buffer (8 mL, pH 8.0) and containing 0.5 M NaCl. After exhaustive washing, 2 mL gel were packed into a Poly-Prep Chromatography Column and equilibrated with 0.15 M NaCl (50 mL). The solution containing IgG (12 mL; 5 mg/mL in 0.15 M NaCl) was loaded onto the column which had been equilibrated for 2 hr at room temperature. After washing with 0.15 M NaCl (60 mL) at 0.2 mL/min, 0.1 M glycine–HCl, pH 2.5 (10 mL), was applied to the column to elute the amanitin-specific antibodies into tubes containing 0.5 M phosphate neutralizing buffer, pH 7.0 (0.4 mL). The antibodies were dialysed against 0.15 M NaCl, concentrated by ultrafiltration, purified further by Sephacryl S-200 superfine (Pharmacia, Uppsala, Sweden) and Detoxi-Gel (Pierce, Rockford, IL, U.S.A.) chromatography, and subjected to SDS–PAGE. Two bands observed at 53 and 26 kDa corresponded to the heavy and light chains, respectively. The equilibrium association constant of the purified amanitin-specific antibody, which was determined by radioimmunoassay [8], was $1.31 \pm 0.6 \times 10^9\ M^{-1}$. Of the plasma IgG, 7.6% was amanitin-specific.

The cytoprotective properties of amanitin-specific polyclonal antibodies were determined in preliminary

* Abbreviations: BSA, bovine serum albumin; IgG, immunoglobulin G.

A.

$(CH_3)_3$-C-O-CO-O-CO-O-C$(CH_3)_3$ + NH_2-$CH_2CH_2NH_2$ → $(CH_3)_3$C-O-CO-NH-$CH_2CH_2NH_2$(BOC-NHCH$_2$CH$_2$NH$_2$)

(I)

B.

BOC-NH-CH_2CH_2-NH_2 + Br-$CH_2CH_2CH_2CH_2CH_2$COOH → Br-$CH_2CH_2CH_2CH_2CH_2$CO-NH-CH_2CH_2NH-BOC + H_2O

(I) (II)

C.

2 CH_3CH_2OH + 2 Na → 2 CH_3CH_2O-Na + H_2↑

$[C_{39}H_{53}N_{10}O_{13}S]$-OH + CH_3CH_2O-Na → $[C_{39}H_{53}N_{10}O_{13}S]$-O-Na + CH_3CH_2OH

α-Amanitin (III)

D.

$[C_{39}H_{53}N_{10}O_{13}S]$-O-Na + Br-$(CH_2)_5$-CO-NH-$(CH_2)_2$-NH-BOC → $[C_{39}H_{53}N_{10}O_{13}S]$-O-$(CH_2)_5$-CO-NH-$(CH_2)_2$-NH-BOC

(III) (II) (IV)

E.

$[C_{39}H_{53}N_{10}O_{13}S]$-O-$(CH_2)_5$-CO-NH-$(CH_2)_2$-NH-BOC → $[C_{39}H_{53}N_{10}O_{13}S]$-O-$(CH_2)_5$-CO-NH-$(CH_2)_2$-$NH_2$

(IV) (V)

F.

$[C_{39}H_{53}N_{10}O_{13}S]$-O-$(CH_2)_5$-CO-NH-$(CH_2)_2$-$NH_2$ + CARRIER-COOH →

(V)

$[C_{39}H_{53}N_{10}O_{13}S]$-O-$(CH_2)_5$-CO-NH-$(CH_2)_2$-NH-CO-CARRIER

(VI)

(I): **tert-butyloxycarbony-ethylenediamine (BOC-ethylenediamine)**
(II): **N-BOC-N′-[6-bromocaproyl] ethylenediamine**
(III): **o-Na-α-Amanitin**
(IV): **o-[5-[[[β-[[t-Butyloxycarbonyl]-amino] ethyl]-amino]-carbonyl]-pent-1-yl]-α-amanitin**
(V): **o-[5-[[Aminoethyl]-amino]-carbonyl]-pent-1-yl]-α-amanitin**
(VI): **α-Amanitin-Linker-Carrier**

Fig. 1. The synthetic pathway for the preparation of the α-amanitin conjugates.

studies in Chang cells (Commonwealth Serum Laboratories, Melbourne, Australia) [9]. These were maintained in Roswell Park Memorial Institute 1640 medium containing 5% fetal calf serum in log growth phase in a 5% CO_2 atmosphere at 37°. The amount of α-amanitin per well was 3 μg, and of amanitin-specific antibodies, 1 mg. Survival rates were assessed by the exclusion of Trypan blue in cells that had been resuspended by trypsin digestion after 36-hr incubation. A partially protective effect of the antibodies was evident at a molar ratio of antibody binding sites to α-amanitin of 4:1 (Table 1).

Discussion

In the preparation of amanitin conjugates to elicit specific antibodies, β-amanitin has been coupled directly to carrier via the aspartic acid residue by several groups [7, 10, 11]. Using these conjugates, we were unable to elicit polyclonal or monoclonal antibodies specific for native amanitin. Based on the structural studies of the amanitins [12, 13], Faulstich *et al.* [5] suggested that this residue is buried in the interior of the molecule and, therefore, attachment to this site will change the conformation of amanitin dramatically. It is known that the conformation of peptide-carrier conjugates plays an important role in the elicitation of their specific antibodies. Due to these conformational restrictions, we synthesized a linker between the carrier and the phenolic hydroxyl group of 6-hydroxytryptophan of α-amanitin. Faulstich used amanitin–BSA conjugates

Table 1. Cytotoxicity of α-amanitin in Chang cells in the presence and absence of amanitin-specific antibodies

	Group	Viable cell numbers (mean ± SD) × 10^{-4} (N = 16)
1	Medium	18.056 ± 5.693
2	Amanitin antibody	20.278 ± 4.070
3	α-Amanitin*	7.333 ± 4.340
4	α-Amanitin + Amanitin antibody†	12.706 ± 2.229

* Indicates significant difference ($P < 0.005$) from groups 1, 2 and 4. Statistical differences between groups were assessed by analysis of variance and the Student–Newman–Keuls test.

† Indicates significant difference ($P < 0.005$) from groups 1, 2 and 3.

with a 14-atom linker to elicit the polyclonal amanitin-specific antibodies that were used in the nylon net immunoassay [6]. We chose a 10-atom linked in the current study because it is a medium length linker which can be synthesized from ethylenediamine and bromohexanoic acid in a one step reaction.

We demonstrated that the amanitin-specific antibodies afforded partial protection from amanitin toxicity in Chang cells. The presumed mechanism of action was by reduction of amanitin uptake by the cells but the partial protection indicates that some uptake of amanitin into cells still occurred. It is also possible that some of the amanitin–antibody complex was internalized by endocytosis and that free amanitin was then released within the cell.

Kirchner and Faulstich reported that amanitin-specific antibodies are hepato-protective *in vivo* [14]. Amanitin-specific rabbit polyclonal antibodies were administered to mice following an intraperitoneal dose of α-amanitin equal to the LD_{50}. They found that the antibodies increased the toxicity of α-amanitin by a factor of 1.9. However, a further study, in which rat-mouse monoclonal antibodies and their Fab fragments were used, demonstrated that the increase in toxicity was due to enhanced amanitin-related nephrotoxicity. Electron microscopic studies showed that the hepatocytes of these animals were completely normal [15]. The nephrotoxicity was probably due to the greater amount of amanitin delivered to the kidneys by the Fab fragments which are eliminated by the kidneys. Thus, the antibodies converted the target organ for toxicity from the liver to the kidney. These authors concluded that antibody therapy was unlikely to be efficacious for amanitin toxicity. However, if the antibody–amanitin complex could be removed by plasmapheresis begun soon after the injection of the antibodies, the nephrotoxicity might be prevented. In the latter case, the use of intact antibody would be preferred over that of Fab fragments because it is confined essentially to the plasma compartment [16].

Acknowledgements—Nian Chen was the recipient of the Ernest Singer Scholarship from the University of Queensland. Supported by the Australian National Health and Medical Research Council.

Department of Medicine
University of Queensland
Princess Alexandra Hospital
Brisbane, QLD 4102
Australia

NIAN CHEN
MARK R. BOWLES
SUSAN M. POND*

REFERENCES

1. Hatfield GM and Brady LR, Toxins of higher fungi. *Lloydia* **38**: 36–55, 1975.
2. Smith TW, Butler Jr VP, Haber E, Fozzard H, Marcus FI, Bremner WF, Schulman IC and Phillips A, Treatment of life-threatening digitalis intoxication with digoxin-specific Fab antibody fragments. *N Engl J Med* **307**: 1357–1362, 1982.
3. Chen N, *Production of Amanitin-Specific Antibodies*. PhD Thesis, The University of Queensland, St Lucia, Queensland, Australia, 1992.
4. Yocum RR and Simons DM, Amatoxins and phallotoxins in *Amanita* species of the Northeastern United States. *Lloydia* **40**: 178–190, 1977.
5. Faulstich H, Trischmann H, Wieland Th and Wulf E, Ether derivatives of α-amanitin. Introduction of spacer moieties, lipophilic residues, and radioactive labels. *Biochemistry* **20**: 6498–6504, 1981.
6. Faulstich H, Zobeley S and Trischmann H, A rapid radioimmunoassay, using a nylon support, for amatoxins from *Amanita* mushrooms. *Toxicon* **20**: 913–924, 1982.
7. Cessi C and Fiume L, Increased toxicity of β-amanitin when bound to a protein. *Toxicon* **6**: 309–310, 1969.
8. Winzor DJ, Bowles MR, Pentel PR, Schoof DD and Pond SM, Adaptation of the Muller method to allow quantitative characterization of the affinity and cross-reactivity of antibodies by competitive radio-immunoassay. *Mol Immunol* **28**: 995–1001, 1991.
9. Chang RS, Continuous subcultivation of epithelial-like cells from normal human tissues. *Proc Soc Exp Biol Med* **87**: 440–443, 1945.
10. Fiume L, Busi C, Campadelli-Fiume G and Franceschi C, Production of antibodies to amanitins as the basis for their radioimmunoassay. *Experientia* **31**: 1233–1234, 1975.
11. Faulstich H, Trischmann H and Zobeley S, A radioimmunoassay for amanitin. *FEBS Lett* **56**: 312–315, 1975.
12. Kostansek EC, Lipscomb WN, Yocum RR and Thiessen WE, Conformation of the mushroom toxin β-amanitin in the crystalline state. *Biochemistry* **17**: 3790–3795, 1978.
13. Tonelli AE, Patel DJ, Wieland Th and Faulstich H, The structure of α-amanitin in dimethylsulfoxide solution. *Biopolymer* **17**: 1973–1986, 1978.
14. Kirchner K and Faulstich H, Purification of amatoxin-specific antibodies from rabbit sera by affinity chromatography, their characterization and use in toxicological studies. *Toxicon* **24**: 273–283, 1986.
15. Faulstich H, Kirchner K and Derenzini M, Strongly enhanced toxicity of the mushroom toxin α-amanitin by an amatoxin-specific Fab or monoclonal antibody. *Toxicon* **26**: 491–499, 1988.
16. Smith TW, Lloyd BL, Spicer N and Haber E, Immunogenicity and kinetics of distribution and elimination of sheep digoxin specific IgG and Fab fragments in the rabbit and baboon. *Clin Exp Immunol* **36**: 384–396, 1979.

* Corresponding author: Prof. S. M. Pond, Department of Medicine, Princess Alexandra Hospital, Ipswich Road, Woolloongabba, Queensland, Australia, 4102. Tel. (61) 7 2405329; FAX (61) 7 2405399.