A RADIOIMMUNOASSAY FOR AMANITIN

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1. Introduction

In the past several attempts [1–4] were made to raise antibodies against the toxins of the dangerous toadstool *Amanita phalloides*. These experiments used the crude extracts of the mushrooms as antigens; however, it appears most unlikely that the toxic peptides, with mol. wts of about 900, exhibit any antigenic properties.

In 1959 Boehringer and Wieland [5] conjugated β-amanitin via the thiophenylester of its carboxylic group to bovine serum albumin and found that the conjugate was too toxic to be used as an antigen in animals. In 1969 Fiume et al. [6,7] prepared the same conjugate using carbodiimides. They found that amanitin bound to albumin had a specific toxicity for protein-consuming cells like the sinusoidal cells of the liver, the macrophages [8], or the cells of the proximal tubules in the kidney [9]. Much less toxic conjugates of amanitin with bovine serum albumin and also dextranes of high mol. wt were obtained by Faulstich and Trischmann [10], who used α-amanitin bound to the macromolecules by a spacer containing an azo-linkage. These compounds, however, were poor antigens, either due to the azo linkage being unstable under physiologic conditions, or the toxin molecule having lost one of its predominant antigenic sites by the chemical modification at the 6-hydroxyindole moiety.

In this situation Fiume et al. decided on the rat for raising antibodies. The rat is less sensitive to amatoxins [11] as well as to amanitin-albumin conjugates [12], than are the rabbits. They succeeded in obtaining a serum, which in a radioimmunoassay enabled the detection 500 pg of amatoxins [12].

In our study we made use of the *N*-hydroxysuccinimidester of β-amanitin, which is better soluble in aqueous protein solutions than is the thiophenylester of the toxin. After the coupling reaction with bovine serum albumin we found 1.3 mol of β-amanitin bound per mole of protein [16]. The resulting amanitin–albumin conjugate was less toxic than that obtained by carbodiimide coupling, since the active ester reaction favors the formation of amide linkages over ester linkages between the toxin and the protein, and besides this, avoids side reactions and oligomerisation of albumin [13]. Both, the presence of ester linkages and an increase in molecular weight probably contribute to the extremely high toxicity of amanitin–albumin conjugates prepared by carbodiimides. Nevertheless, the toxicity in the white mouse of the resulting derivative was still 3–4 times higher than that of β-amanitin itself. This, however, was overcome by a covalent attachment of the peptide-protein-complex to polylysine. The amanitin–albumin–polylysine conjugate was mixed with Freund's complete adjuvant and administered to rabbits by intradermal application. Though many of the animals died, and though the titer of amanitin-binding proteins in the sera of the surviving animals remained low, a radioimmunoassay could be established. By the use of [^{3}H]*O*-methyl-demethyl-γ-amanitin [14,15] with a specific activity of 2.4 Ci/mmol we succeeded in detecting as little as 50 pg of α-amanitin.

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$$[C_{38}H_{52}N_9O_{13}S]\text{-}COOH \xrightarrow[\text{in DMF}]{N(C_2H_5)_3,\ ClCOOCOOC_2H_5,\quad HO\ N(COCH_2\text{-})_2} [C_{38}H_{52}N_9O_{13}S]\text{-}COON(COCH_2\text{-})_2$$

$$[C_{38}H_{52}N_9O_{13}S]\text{-}COON(COCH_2\text{-})_2 \xrightarrow[\text{pH 7–9}]{\text{bovine serum albumin}} [BSA]\text{-}NHCO\text{-}[C_{38}H_{52}N_9O_{13}S]$$

$$[BSA]\text{-}NHCO\text{-}[C_{38}H_{52}N_9O_{13}S] \xrightarrow[\text{pH 7}]{\text{polylysine, } HCO(CH_2)_3CHO} [Lys]_n\text{-}N = CH(CH_2)_3CH = N\text{-}[BSA]\text{-}NHCO\text{-}[C_{38}H_{52}N_9O_{13}S]$$

$[C_{38}H_{52}N_9O_{13}S]$-COOH : β-amanitin
[BSA] : bovine serum albumin
$[Lys]_n$: polylysine, mol. wt 85 000, n ~ 660
DMF : dimethylformamide

2. Materials and methods

2.1. *Conjugation of β-amanitin-bovine serum albumin [16] to polylysine and immunisation of the rabbits*

60 mg Polylysine HBr (Sigma) (mol. wt 85 000) in 4 ml of 1 mM $NaHPO_4$ buffer pH 7.0 were reacted with 1.6 ml of 2.5% glutardialdehyde under slow stirring for 1 hr at 20°C. After addition of a drop of *n*-octanol, the solution of 160 mg of a β-amanitin–bovine serum albumin conjugate [16] (molar ratio amanitin/albumin = 1.3) in 1.6 ml of the phosphate buffer was added under vigourous stirring. The fine precipitate, suspended in 4 ml of 0.9% NaCl was homogenized with 4 ml Freund's complete adjuvant.

8 Female New Zealand rabbits of 2–3 kg body weight received intradermal injections at about 30 points in the neck, into the inquinal folds and into the interdigital membranes corresponding to 230 μg amanitin/kg body weight. After a time of rest of 7 weeks the first booster was administered intramuscularly, corresponding to about 70 μg amanitin/kg body weight. Serum was taken from a vein in the ear of the rabbit generally one week after each booster injection, which were given in 2 week-intervals.

2.2. *[^{3}H]O-methyl-demethyl-γ-amanitin*

20 mg (22 μmol) *O*-methylaldoamanitin were dissolved in 1.5 ml H_2O, cooled to 4°C and treated, under magnetic stirring, with 1.9 mg (50 μmol) of [^{3}H]$NaBH_4$ (spec. act. 9.9 Ci/mmol) for 1.5 hr. After addition of 2.5 ml 0.1 N HCl the reaction mixture was kept acidic for 20 min, neutralized with 0.1 N NaOH and kept under a stream of N_2 for 3 hr. After evaporation to dryness and application to a Sephadex LH-20 column (2.5 × 250 cm) 10.8 μg (54%) of the tritiated compound was eluted in the fraction 423 ml–528 ml, as the second of two large peaks. The specific activity of the toxin was 2.4 Ci/mmol. R_f = 0.35 in *sec*-butanol–ethylacetate–water (14:12:5), as a single compound on a silica thin-layer plate monitored by a scanner and by the absorption of u.v. light. The compound was stored in methanol at the temperature of liquid N_2.

2.3. *Radioimmunoassay of α-amanitin*

To 200 μl of a buffer at 4°C (0.05 M borate, 0.05 M KCl, pH 8.0, 0.1% lysozyme, Serva) in Eppendorf vessels 1.7 pmol of [^{3}H]*O*-methyl-demethyl-γ-amanitin (5200 cpm) in 30 μl of the same lysozyme-borate buffer were added followed by the addition of 30 μl of α-amanitin in a dilution series. After the addition of 30 μl of the diluted serum (1:20) incubation was allowed for 16 hr at 4°C.

A γ-globulin-borate buffer (0.05 M borate, pH 8, 0.06% bovine γ-globulin (Serva) was prepared and mixed either with charcoal (Norit A, Serva) (5 g/80 ml) or Dextran T 70 (Pharmacia) (100 mg/80 ml). After the incubation was finished, 30 μl of a mixture

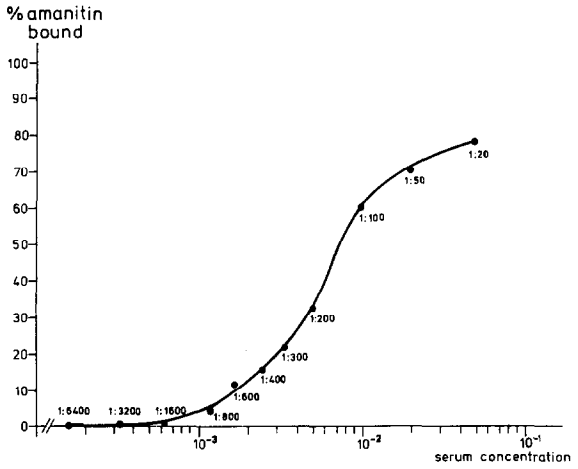

Fig.1. Titer of amanitin binding proteins in the serum of a rabbit. Final dilution of the serum in the assay plotted versus % of radioactive amanitin bound (100% = 1.7 pmol-[^{3}H]*O*-methyldemethyl-γ-amanitin = 5200 cpm).

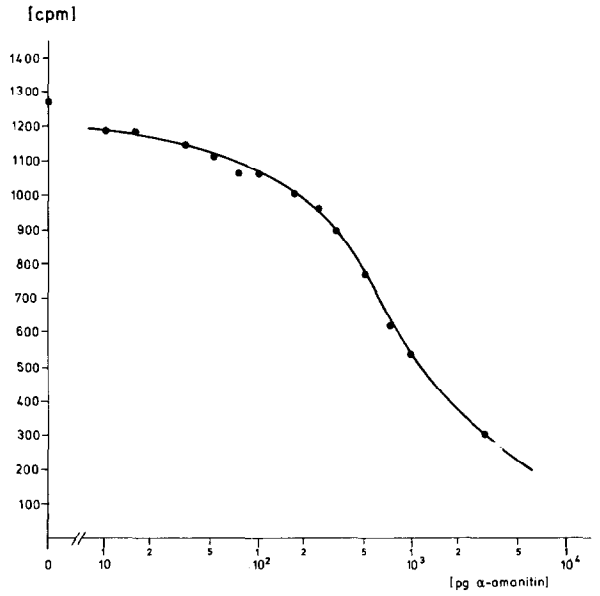

Fig.2. Typical calibration curve for α-amanitin obtained with a final dilution of serum in the assay of 1:200 (control:3800 cpm).

charcoal/dextrane (1 + 9) was added. After 20 min of vigorous shaking at 4°C the charcoal was spun down, and 200 μl of the supernatant was counted.

3. Results and discussion

The binding procedure of β-amanitin-bovine serum albumin to polylysine had detoxified the conjugate at least 3–4-fold. Nevertheless, the injection of antigenic material equivalent to 0.23 mg β-amanitin/kg body weight nearly corresponded to the LD_{50}-value, since 4 of the 8 rabbits treated died within 1 week.

All the surviving animals had developed antibodies against the toxin 1 week after the first booster injection; the concentration of amanitin-binding proteins, however, remained low, and still decreased rapidly after the following booster injections. In one animal for example we measured that after the third booster the concentration of antibodies was only 16% of the value after the first booster. After a fourth booster injection the conditions of the animals grew worse: 2 of 4 animals died, the 2 others had to be killed.

The serum, which was taken 1 week after the first booster, bound 50% of 1.7 pmol of tritiated amanitin at a final dilution of 1:150. This value is derived from the titer curve in fig.1. In a final dilution of 1:200 this serum was used to detect at least 50 pg of α-amanitin, as shown in the calibration experiment in fig.2. For practical use it may be crucial to find out the severity of an human intoxication much earlier than under the optimum conditions described above. Therefore we also tried an assay at 25°C with the incubation time reduced to 45 min. Provided all solutions were prepared before the assay was started the results were obtained only 3 hr later with, however, a 5 times lower sensitivity. Two other toxins, which occur in the mushroom, β- and γ-amanitin, were bound to exactly the same extent as α-amanitin.

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