Epidermal Growth Factor Labeled β-Amanitin–Poly-L-ornithine: Preparation and Evidence for Specific Cytotoxicity†

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Received November 27, 1989; Revised Manuscript Received April 4, 1990

ABSTRACT: Poly-L-ornithine with an average molecular weight of 32K was reacted with β-amanitin hydroxysuccinimide ester to form an amide-linked toxin conjugate. Loading of the polymeric chain with amanitin was high, corresponding to up to 35% of the total weight. To this amatoxin vehicle we attached a targeting molecule, human recombinant leucine-21 epidermal growth factor ($hrEGF_L$), via a disulfide-containing linker moiety. A typical average stoichiometry of the $hrEGF_L$ labeled toxin conjugate was $(L\text{-Orn})_{164}(\beta\text{-amanitin})_{19}(COC_2H_4SSC_2H_4CO\text{-}hrEGF_L)_2$. The affinity for EGF receptors of $hrEGF_L$ bound in this conjugate was tested by using A 431 cells. The affinity was eight times lower than that of unsubstituted $hrEGF_L$ but regarded as high enough for studying specific toxicity effects with cells bearing EGF receptors. We found that β-amanitin in the labeled conjugate was able to inhibit the growth of A 431 cells at a concentration of 28 nM, 80 times lower than for native β-amanitin and 20 times lower than for poly-L-ornithine-bound β-amanitin without the $hrEGF_L$ label. The approximately 20-fold enhancement of cytotoxicity suggests a specific internalization of the toxin conjugate mediated by the hormone label. This idea is supported by the fact that also in another transformed fibroblast cell line, with an increased though smaller number of EGF receptors than A 431 cells, the corresponding enhancement of cytotoxicity was demonstrable but less pronounced (7-fold). The hormone-mediated increase in cytotoxicity of EGF labeled poly-L-ornithine–β-amanitin conjugates, combined with their moderate toxicity in the mouse, encourages further examination of such compounds in tumor model systems in vivo. In general, poly-L-ornithine spiked with β-amanitin may find wider application as the toxic component in chimeric toxin complexes.

Amatoxins act as hepatotoxins mainly through the presence in hepatocytes of a specific transporting system that under physiological conditions carries bile acids and various xenobiotics (Kröncke et al., 1986; Münter et al., 1986). Cells lacking this transporting system appear well protected from amatoxins; however, all kinds of mammalian cells are susceptible to amatoxins, provided the toxins are able to pass through the plasma membrane. This can occur through the attachment of amatoxins to macromolecules that are internalized via endocytosis. Thus amatoxins covalently bound to proteins are highly toxic to protein-consuming cells, such as sinusoidal cells of liver (Derenzini et al., 1973) or macrophages (Fiume & Barbanti-Brodano, 1974). While amatoxin conjugates were shown to damage sinusoidal cells of liver, leaving most of the hepatocytes intact, the lesions observed were of the same kind as those induced by free amatoxins in hepatocytes (Marinozzi & Fiume, 1971). This indicates that by appropriate chemical modification one can indeed divert the amatoxins from their normal target cells and cause them to direct their toxic activities to other cells, for example, to cells involved in protein-clearing processes.

Destruction of sinusoidal cells of liver is of course undesirable and leads to a high in vivo toxicity of such compounds. For example, β-amanitin linked to bovine serum albumin showed a toxicity 50–100 times higher than that of the free toxin (Bonetti et al., 1970). Similarly α-amanitin, when complexed to amatoxin-binding immunoglobulins or Fab fragments, was up to 50 times more toxic for mice than α-amanitin in the absence of such proteins (Faulstich et al., 1988). From these data we concluded that internalization of amatoxins via macromolecular derivatives must be confined to endocytotis mediated by a ligand–receptor-dependent transporting system. Choice of the ligand or targeting molecule would allow one to select for the kind of cells to be eliminated. Clearly, ligands with high affinity for their receptors can be expected to mediate internalization at concentrations lower than those at which unspecific endocytotis may occur.

Epidermal growth factor (EGF) has a K_D of 10^{-9}–10^{-11} M (King & Cuatrecasas, 1982) and seems particularly suitable for this purpose. In addition, it is known that the internalized EGF receptor complex takes the lysosomal pathway (Willingham & Pastan, 1982), a fact that is most important for achieving the intracellular release of the toxin. EGF has been used as a signal protein in conjugates with ricin A (Cawley & Herschman, 1980), diphtheria toxin (Simpson et al., 1982), and *Pseudomonas* exotoxin (Lyall et al., 1987). The conjugates were studied for their specific cytotoxic activity against various cell lines (Banker et al., 1989; Banker & Herschman, 1989). For the ricin conjugates the role of the binding proteins (ricin B or EGF) has been investigated in detail (Herschman, 1984), as well as the internalization of the EGF–toxin complex and the onset of toxic activity (Shimizu et al., 1984). Other experiments of this kind used—instead of EGF—antibodies aginst the EGF receptor conjugated with, for example, ricin A (Vollmar et al., 1987), doxorubicin (Aboud-Pirak et al., 1989), or gelonin (Ozawa et al., 1989).

In the present study we coupled human recombinant leucine-21-substituted EGF ($hrEGF_L$),[1] linked to a thiol-capturing

† This work was supported by the Deutsche Forschungsgemeinschaft (Fa-126/7-1).
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[1] Abbreviations: CAI, isobutyl chloroformate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; HBSS, Hank's balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOSUC, *N*-hydroxysuccinimide; $hrEGF_L$, human recombinant leucine-21 epidermal growth factor; L-Orn, L-ornithine; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; PDP-, (2-pyridyldithio)propionyl-; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; THF, transformed human fibroblasts; TNBS, trinitrobenzenesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/90/0429-6839$02.50/0

moiety (PDP), to a β-amanitin-spiked poly-L-ornithine, in which residual amino side chains were modified as thiol residues, thus allowing the toxin vehicle to be linked with the signal protein by formation of a disulfide bridge. The $hrEGF_L$ labeled toxin conjugate was tested for its in vitro toxicity to two tumor cell lines possessing a large number of EGF receptors, as well as in vivo for its toxicity in the mouse.

Materials and Methods

Poly-L-ornithine hydrogen bromide (32 kDa), *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and *N*-ethylmaleimide (NEM) were from Sigma, St. Louis. Isobutyl chloroformate (CAI) was from Aldrich, Steinheim; *N*-hydroxysuccinimide (HOSUC) from Fluka, Buchs; and 2-mercaptoethanol from Roth, Karlsruhe. All reagents were analytical grade. Cleland's Reductacryl TM-Reagent was purchased from Calbiochem, GmbH, Frankfurt; CM-Sephadex C-25, DEAE-Sephadex A-50, and Sephadex LH-20 were from Pharmacia Uppsala. Human recombinant leucine-21 epidermal growth factor ($hrEGF_L$) was from PROGEN Biotechnik, Heidelberg, and [^{125}I]$hrEGF_L$ (specific activity, 900 Ci/mmol) from Amersham Buchler, Braunschweig. A 431 cells and SV80 transformed human fibroblasts (THF) were provided by Dr. W. W. Franke of the German Cancer Research Center, fetal calf serum (FBS) and amphothericin B were bought from Biochrom KG, Berlin, and streptomycin/penicillin was a product of Boehringer, Mannheim.

Purification of β-Amanitin. β-Amanitin was isolated from aqueous extracts of *Amanita phalloides* mushrooms by repeatedly passing the evaporated material through columns of Sephadex LH-20 (5 × 250 cm) developed with water (0.02% sodium azide) (Faulstich et al., 1972). The fraction containing the toxin was identified by spectrometry, applied to a column of DEAE-Sephadex A-50 (5 × 18 cm), and eluted with a gradient of acetic acid (0.1–0.5%). The toxin was 90% pure by tlc (2-butanol–acetic acid–water, 4:1:1); yield, 55 mg/kg of fresh mushroom tissue.

Poly-L-ornithine–β-Amanitin (I). For coupling with poly-L-ornithine the carboxylic group of β-amanitin was activated as mixed isobutylcarbonic anhydride. For this, 10 mg of β-amanitin (11 μmol), dried in vacuo over P_4O_{10}, was dissolved in 1 mL of dry, freshly distilled dimethylformamide. The pH was adjusted to 8.5 by the addition of dry triethylamine. After the mixture was cooled to −15 °C, 2.3 μL (16 μmol) of CAI was added, and the mixture was stirred at this temperature for 30 min. Then 2.5 mg (22 μmol) of HOSUC dissolved in 0.01 mL of dimethylformamide was added and the mixture was allowed to react for a further 2 h at room temperature. The reaction mixture was added to a solution of 21 mg of poly-L-ornithine hydrogen bromide (32 kDa, 110 μmol of ornithine) in 1 mL of H_2O adjusted to pH 7.5 by the addition of 1 N NaOH and kept overnight at room temperature while gently stirring. Uncoupled β-amanitin was separated by passing the reaction mixture through a Sephadex G-25 column (1.7 × 50 cm) equilibrated with 0.9% NaCl. The fraction eluting at 15 mL was characterized for its content of β-amanitin ($\epsilon_{310} = 1.35 \times 10^4$ M^{-1} cm^{-1}) and free amino groups were determined as picrylate after reaction with TNBS. Yield (related on β-amanitin) was 50%. The product was lyophilized and stored at 20 °C.

Derivatization of the Poly-L-ornithine–β-Amanitin with the Cross-Linking Reagent SPDP (II). Ten milligrams of lyophilized compound I (containing 9 μmol of polymerized L-ornithine, 1 μmol of amanitin, and 120 μmol of NaCl) was dissolved in 3 mL of 0.1 M Tris-HCl buffer (pH 8) and 0.4 mg (1 μmol) of SPDP dissolved in 0.1 mL of freshly distilled dimethylformamide were added. The mixture was stirred for 1 h at 20 °C. Compound II was separated from unreacted cross-linker by chromatography on a Sephadex G-25 column (1.7 × 50 cm) with 0.9% NaCl. The (2-pyridyldithio)-propionate residues introduced into the polymer were determined by cleaving the 2-thiopyridine with excess 2-mercaptoethanol and measuring the increase in absorbance at 340 nm ($\epsilon_{340} = 8.5 \times 10^3$ M^{-1} cm^{-1}). Yield was 60% (related to the amount of SPDP). The product was lyophilized and stored at 20 °C.

Introduction of SPDP into $hrEGF_L$ (IV). $hrEGF_L$, 0.5 mg (0.08 μmol), was dissolved in 1 mL of 0.1 M Tris-HCl, pH 8.0, and reacted, at room temperature and with magnetic stirring, with 0.2 mg (0.64 μmol) of SPDP dissolved in 0.05 mL of freshly distilled dimethylformamide. Isolation and characterization were as for compound II. Yield was 60% related to $hrEGF_L$. The product IV was stored in the lyophilized state at 4 °C.

Preparation of $hrEGF_L$–Poly-L-ornithine–β-Amanitin (V). Twenty milligrams of the lyophilizate of compound (II) (containing 0.9 μmol of polymerized L-ornithine, 0.1 μmol of amanitin, 0.3 μmol of (2-pyridyldithio)propionate, dissolved in 3 mL of 0.1 M Tris-HCl, pH 7.5) was reacted with 20 mg (ca. 100-fold excess) of Cleland's Reductacryl TM until the increase in absorbance at 340 nm showed that the reduction was finished. The gel beads were removed by centrifugation and the supernatant containing compound III with 0.3 μmol of SH was added immediately to a solution of compound IV (0.03 μmol) in 3 mL of 0.1 M Tris-HCl, pH 8.0. The coupling reaction was again monitored by absorbance at 340 nm and found to be complete after 20 min. At that time 25 μg (0.2 μmol) of NEM was added to block the free SH groups. After 2 h at 4 °C the pH of the solution was adjusted to 5.0 by adding 0.1 N HCl.

For separation from unreacted IV, the mixture (6–7 mL) was diluted with 28 mL of 0.2% NaCl and treated with 250 mg of CM-Sephadex C-25 suspended in 2 mL of H_2O. After the mixture was gently shaken for 15 min, the gel beads were removed by centrifugation. Desorption of compound V was achieved by vortexing the pellet with 35 mL of 2 M NaCl. After removal of the resin by centrifugation, the supernatant was characterized spectrophotometrically and directly used in cell culture experiments. Yield was 20%.

Preparation of $hrEGF_L$–Poly-L-ornithine. Preparation of this control substance followed the same procedure starting from poly-L-ornithine hydrogen bromide instead of its β-amanitin derivative (I).

Cell Cultures. A 431 cells and SV80 transformed human fibroblasts (THF cells) were grown in an atmosphere of 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 1 unit/mL penicillin, 1 μg/mL streptomycin, and 2.5 μg/mL amphothericin B.

Determination of the Affinity of the $hrEGF_L$ Label in Compounds IV and V to EGF Receptors. A 431 cells were grown to a density of 1×10^6 cells per 35-mm dish and washed twice with 1 mL of warm Hank's balanced salt solution (HBSS). The cells were incubated for 1 h at 37 °C with 1 mL of DMEM containing 20 mM HEPES, pH 7.5, and 1.5 mg of [^{125}I]$hrEGF_L$ (0.9 μCi/pmol) together with compounds IV and V and poly-L-ornithine–$hrEGF_L$ in concentrations corresponding to 30–700 ng of bound $hrEGF_L$. After they were washed with 1 mL of ice-cold HBSS the cells were solubilized in 1 mL of N NaOH for 1 h at 37 °C and counted.

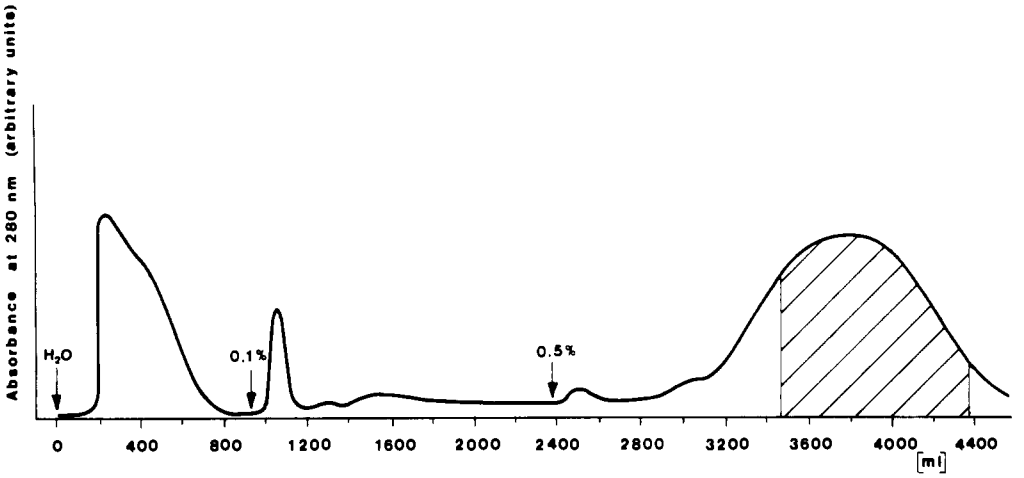

FIGURE 1: Elution diagram of the final purification of β-amanitin on DEAE–Sephadex A-50. Elution was monitored by absorbance at 280 nm. With water and 0.1% acetic acid, undefined impurities were eluted, while the first fractions eluted with 0.5% acetic acid contained acidic phallotoxins.

Cells incubated with [^{125}I]hrEGF$_L$ alone were taken as the 100% value.

Determination of the Number of EGF Receptor on A 431 and THF Cells. A 431 and THF cells were grown to a density of 1 × 10^6 cells per 35-mm dish and washed twice with 1 mL of warm HBSS. A 431 and THF cells were incubated in 1 mL of DMEM with 20 mM HEPES, pH 7.5, containing 6–200 ng of [^{125}I]hrEGF$_L$ (specific activity 0.5–28 nCi/pmol) or 1–100 ng [^{125}I]hrEGF$_L$ (specific activity 1.6–63 nCi/pmol), respectively, for 4 h at 4 °C. If a 1:1 binding ratio and approximately 10^6 receptors per A 431 cell and 10^5 receptors per THF cell are assumed, the labeled hormone was in excess of 27-fold or 75-fold, respectively. The cells were washed once with 1 mL of HBSS, detached from the dishes by 10-min incubation with 1 mL of 0.15% trypsin, and transferred to counting tubes.

Determination of Cytotoxic Activities. A 431 and THF cells were grown overnight to a density of 1 × 10^5 cells per 35-mm dish. Toxin conjugates and control substances were added in various concentrations in a small volume. After 24 h the medium containing the toxins was removed and fresh medium added. After further culture periods of 24, 48, and 72 h, the number of the surviving cells was determined by washing with PBS, incubation (10 min) with a mixture of 0.15% trypsin in PBS and 0.9% Trypan Blue in H_2O (2:1), and counting of the cells excluding Trypan Blue in a Neubauer chamber. IC_{50} values were calculated on the basis of the numbers of cells surviving 96 h of culturing.

In Vivo Toxicity. Solutions of β-amanitin and β-amanitin conjugates in 0.9% NaCl were prepared under spectrophotometric control. Doses of 0.04–2.0 mg of amanitin/kg bwt were administered to female black mice (strain C_3He, ca. 25 g) into the tail vein (100 μL/10 g bwt). As a control, poly-L-ornithine HBr was administered analogously in doses of 2.0–20 mg/kg bwt, which is the concentration range of poly-L-ornithine in the conjugates.

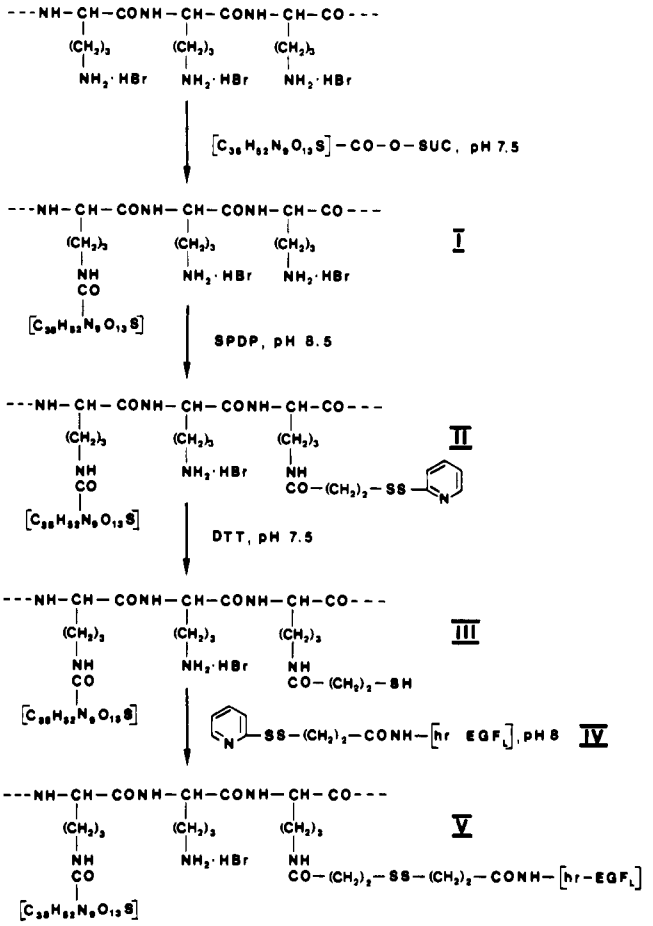

FIGURE 2: Reaction scheme of the preparation of the β-amanitin conjugate of poly-L-ornithine and its coupling with hrEGF$_L$. In the first and second step, β-amanitin and PDP residues are introduced into poly-L-ornithine. After reduction with DTT, the PDP derivative of hrEGF$_L$ is linked with the toxin vehicle.

RESULTS

Repeated runs of *Amanita phalloides* mushroom extracts on Sephadex LH-20 followed by ion-exchange treatment yielded β-amanitin with a purity of >90% (Figure 1) and in amounts large enough that different chemical methods for coupling it to poly-L-ornithine could be explored. It was convenient to activate the carboxylic group of the toxin as a mixed anhydride (Wieland & Boehringer, 1960) and convert it to its *N*-hydroxysuccinimide ester (Figure 2) before transferring it to the aqueous solution containing poly-L-ornithine. After separation of the unreacted toxin by gel filtration, the β-amanitin–poly-L-ornithine conjugates were characterized by the molar absorption of the toxin (Figure 3a) and TNBS titration of free amino groups and were found to be [L-orn]$_{164}$[β-amanitin]$_n$ for n = 5–19. The highest amanitin load corresponds to a toxin content of the conjugate of 35% by weight. By comparison, conjugates of β-amanitin with proteins like bovine serum albumin had yielded maximum amatoxin

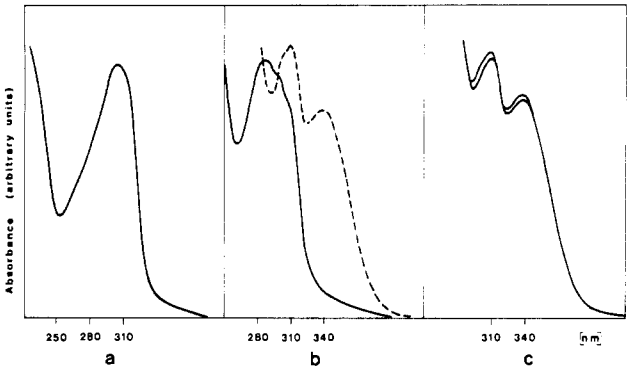

FIGURE 3: UV spectrum of (L-Orn)$_{164}$(β-amanitin)$_{19}$ (a) and PDP-(L-Orn)$_{164}$(β-amanitin)$_{19}$ (b, solid line) and after deblocking with excess mercaptoethanol or Reductacryl (b, dashed line). Change of absorbance at 340 nm caused by coupling of PDP-hrEGF$_L$ to SH-(L-Orn)$_{164}$(β-amanitin)$_{19}$ (c).

contents of 3% of the total (Faulstich & Fiume, 1985).

The NH_2 group of human recombinant leucine-21 epidermal growth factor (hrEGF$_L$) was reacted with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) to yield the 1:1 adduct in 60% yield. By a similar reaction (2-pyridyldithio)propionyl (PDP) residues were introduced into the β-amanitin–poly-L-ornithine conjugate and deblocked by reaction with an excess of resin-supported dithioerythrol. The resulting thiol derivative of the polymer was not isolated but reacted in situ with the PDP-hrEGF$_L$. In order to avoid loss of the hormone label by intramolecular disulfide exchange, excess SH was blocked with NEM.

Characterization of PDP-hrEGF$_L$ and PDP-L-ornithine–β-amanitin was achieved spectrophotometrically by measuring the amount of 2-thiopyridine anion at 340 nm released upon the addition of excess mercaptoethanol (Figure 3b). By the same method we monitored the final coupling reaction and found the formation of the disulfide to be complete after 20 min (Figure 3c). For purification, particularly for removal of unreacted (2-pyridyldithio)propionyl-hrEGF$_L$ (80%), the reaction mixture was treated with CM-Sephadex C-25, which binds poly-L-ornithine and its derivatives, but not (2-pyridyldithio)propionyl-hrEGF$_L$. Desorption of the reaction product was achieved with 2 M NaCl. A typical purified, hormone-labeled β-amanitin–poly-L-ornithine conjugate (compound V) that was used in the biological assays had the average stoichiometry (L-Orn)$_{164}$(β-amanitin)$_{19}$-($COC_2H_4SSC_2H_4CO$-hrEGF$_L$)$_2$.

A 431 cells were used for studying the various hrEGF$_L$ derivatives for their ability to displace [^{125}I]hrEGF$_L$ from its receptor. While PDP-hrEGF$_L$ exhibited, within the limit of error, the same binding capacity as hrEGF$_L$ (50% displacement at 13 nM) (Figure 4), the affinity of the hormone obviously decreased in the macromolecular derivatives with increasing molecular weight, to a 50% value of 30 nM (hrEGF$_L$–poly-L-ornithine) or 100 nM (compound V, hrEGF$_L$–poly-L-ornithine–β-amanitin). However, considering a K_D of approximately 0.1 nM of native hrEGF$_L$ to its receptor, an 8-fold decrease of hrEGF$_L$ in compound V appeared tolerable for the intended biological studies.

As shown by others (Fabricant et al., 1977) and confirmed in this study, A 431 cells possess a high density of EGF receptors on their surface (up to 2×10^6 per cell) and hence appear exceptionally well suited for detecting cytotoxicity that is mediated by specific uptake of the toxin conjugate via its hrEGF$_L$ label. In these cells free β-amanitin inhibited growth with an IC_{50} of 2.2 μM, a value close to that determined previously for other cultured cells, for example, Epstein Barr

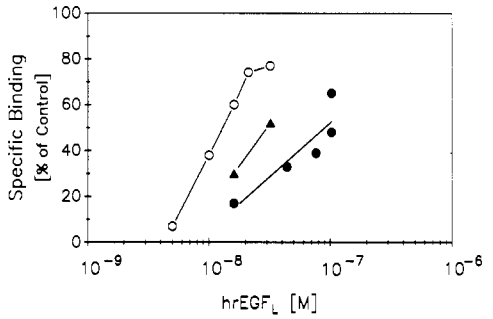

FIGURE 4: Displacement of [^{125}I]hrEGF$_L$ from A 431 cells by various hrEGF$_L$ derivatives: hrEGF$_L$ or PDP-hrEGF$_L$ (○), (L-Orn)$_{164}$-(hrEGF$_L$)$_2$ (▲), and (L-Orn)$_{164}$(β-amanitin)$_{19}$(hrEGF$_L$)$_2$ (●). One hundred percent specific binding was calculated as the difference between total binding and binding of [^{125}I]hrEGF$_L$ in the presence of 100-fold excess of hrEGF$_L$.

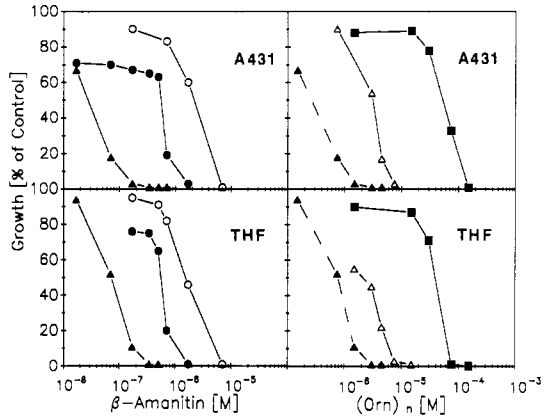

FIGURE 5: Growth inhibition of A 431 cells and THF cells by (L-Orn)$_{164}$(β-amanitin)$_{19}$(hrEGF$_L$)$_2$ (▲), β-amanitin (○), (L-Orn)$_{164}$-(β-amanitin)$_{19}$ (●), (L-Orn)$_{164}$(hrEGF$_L$)$_2$ (△), and poly-L-ornithine (■) as dependent on the concentrations of β-amanitin (free or bound) or L-ornithine. Each point is the average of five independent experiments performed in triplicate culture dishes.

virus transformed lymphocytes (5 μM; C. Wilbertz, B. Ungemach, and H. Faulstich, unpublished results). Bound to the polymeric support and labeled with hrEGF$_L$ (compound V), β-amanitin caused a 50% growth inhibition at a toxin concentration of only 0.028 μM, which is 80 times lower than that of the free toxin. For the toxin conjugate without hormone label we found the corresponding IC_{50} value to be 0.58 μM (Figure 5). This value was not changed when in this experiment free hrEGF$_L$ was added (data not shown). The data indicate that hrEGF$_L$ caused a 20-fold increase of cytotoxicity, but only when the hormone label was covalently attached to the conjugate. Since basic poly(amino acid)s themselves exhibit cytotoxic activities we also determined for A 431 cells the IC_{50} value of poly-L-ornithine (32 kDa), which was found to be 54 μM. Remarkably, labeling of the poly-L-ornithine with hrEGF$_L$ enhanced this toxicity also, by a factor of 17 (IC_{50} = 3.2 μM, Figure 5).

Corresponding cytotoxicity studies were performed with another cell line, SV80-transformed human fibroblasts (THF), which have only 1×10^5 EGF receptors per cell. In this cell line compound V caused a 50% growth inhibition at 0.08 μM, i.e., 20 times lower than the IC_{50} of free β-amanitin (1.6 μM) and seven times lower than the concentration determined for the toxin conjugate without hormone label (0.56 μM). For the carrier alone, poly-L-ornithine, we found an IC_{50} value of 40 μM in the presence as well as in the absence of added hrEGF$_L$. However, also in this cell line hrEGF$_L$, when covalently attached to poly-L-ornithine, enhanced its toxicity by about 10-fold (IC_{50} = 2.8 μM, Figure 5).

Table I: Toxicity of β-Amanitin, Three β-Amanitin–Poly-L-ornithine Conjugates, Compound V, and Poly-L-ornithine (32 kDa) in the Female Black Mouse (C_3He)

compound	doses (mg/kg body wt)		animals died/animals treated	death after days	LD_{50} (mg/kg body wt)
	amatoxin	$(L\text{-orn}\cdot HBr)_n$			
β-amanitin	2		2/2	3	
	1		3/3	3–5	0.70
	0.4		0/3		
$(L\text{-orn–}\beta\text{-amanitin})_5\text{–}(L\text{-orn}\cdot HBr)_{159}$	2	14	1/1	1	
	0.2	1.4	2/2	4	0.137
	0.1	0.7	1/3	4	
	0.04	0.28	0/3		
$(L\text{-orn–}\beta\text{-amanitin})_{10}\text{–}(L\text{-orn}\cdot HBr)_{154}$	2	7	1/1	1	
	0.2	0.7	2/2	4	0.10
	0.1	0.35	2/3	4	
	0.04	0.14	0/3		
$(L\text{-orn–}\beta\text{-amanitin})_{18}\text{–}(L\text{-orn}\cdot HBr)_{146}$	2	3.9	1/1	1	
	0.2	0.39	2/2	3	
	0.1	0.2	3/3	4–5	0.07
	0.04	0.078	0/3		
compound V	2	3.7	1/1	1	
$(L\text{-orn–hrEGF})_2\text{–}(L\text{-orn–}\beta\text{-amanitin})_{19}\text{–}(L\text{-orn}\cdot HBr)_{143}$	1	1.8	0/2		1.50
	0.4	0.74	0/1		
	0.2	0.37	0/2		
$(L\text{-orn}\cdot HBr)_{164}$		20	2/2	1	
		10	2/3	1	10.0
		4	0/3		
		2	0/2		

As an initial measure of the in vivo toxicity of the β-amanitin derivatives, we determined the LD_{50} value of $hrEGF_L$-labeled β-amanitin poly-L-ornithine (compound V) in the black mouse. We likewise determined the toxicities of various poly-L-ornithine–β-amanitin conjugates lacking the label, but possessing different loads of β-amanitin (n = 5, 10, 19). As controls we measured also the LD_{50} values of β-amanitin and poly-L-ornithine (32 kDa). The results are shown in Table I.

Discussion

In contrast to their wide use as a tool in molecular biology [for a review, see Faulstich (1980)], only a few attempts have been made so far to employ amatoxins in toxin targeting experiments. One reason for this may be that the toxic peptides were not available in amounts large enough for sampling chemical modifications. On the other hand, amatoxins bound to macromolecules were found to be extremely toxic in vivo and thus thought to have only limited biological application. This study is the first attempt at tailoring an amatoxin-spiked vehicle labeled with a signal protein that can exhibit specific toxicity to distinct cells, in concentrations probably low enough not to damage existing clearing systems of mammalian organisms.

Acidic β-amanitin is present in *Amanita phalloides* toadstools in amounts similar to α-amanitin (Wieland & Faulstich, 1983). Unlike the neutral α-toxin, which needs derivatization with a functional spacer (Faulstich et al., 1981), β-amanitin can be coupled directly to amino groups of proteins or basic poly(amino acid)s via its carboxylic group. In poly-L-lysine (30–165 kDa), for example, one out of 12–18 lysine residues could be substituted by β-amanitin (Faulstich et al., 1975); however, the conjugate showed properties that were unfavorable for its biological use: low solubility and a shift of the pK value of the (ind-6)-hydroxy group in β-amanitin from 9.3 to 7.2 that accelerated oxidative degradation of the toxin. This "enzymoid" effect was absent when poly-L-ornithine (32 kDa) was used as a carrier, the conjugate combining stability of the attached amatoxin with good solubility in aqueous buffers. On average, one out of eight ornithine residues could be modified with amanitin. Unmodified amino groups were reserved for the attachment of the hormone label or for a possible intracellular degradation of the conjugate by trypsin-like proteases.

Amatoxins seem to be particularly suitable as carrier-bound toxins because they withstand proteolytic degradation of their carriers. Hitherto, no mammalian enzyme has been found that would be able to metabolize the cyclic peptides. One would expect therefore that amatoxins retain their full biological activity inside the cell provided they are released from the carrier. Proteolytic breakdown of the carrier seems important because amatoxins bound to macromolecules possess only approximately 1% of the capacity of the free toxins to inhibit RNA polymerase II (Faulstich & Fiume, 1985). We found that medium concentrations of our amanitin complex in the nanomolar range were able to inhibit the growth of A 431 cells. This suggests to us that the major portion of the β-amanitin must indeed have been cleaved from its polymeric support. It is unclear, however, whether hydrolytic degradation of the carrier released native β-amanitin or a low molecular weight derivative of it.

There are several examples in the literature where SPDP was used to introduce thiol scavenger units (PDP) into proteins (Carlsson et al., 1978; Gros et al., 1985) that subsequently could be reacted either with native protein SH groups as in ricin A (Cawley & Herschman, 1980; Olsberg et al., 1985) or with SH groups likewise introduced into proteins via the PDP moiety (Colombatti et al., 1983; Herschman, 1984; Singh et al., 1989; Conde et al., 1989). The PDP derivative of EGF has been described previously (Cawley and Herschman, 1980). In the present study this derivative was reacted with a poly-L-ornithine–β-amanitin conjugate bearing an approximately 10-fold excess of 3-mercaptopropionate moieties. By spectrophotometric monitoring we found that $hrEGF_L$–PDP could be reacted with the poly-L-ornithine conjugate with a 20% yield forming compound V with an average carrier:toxin:label ratio of 164:19:2. One should bear in mind that compound V represents a population of macromolecules corresponding to the commercially available mixture of poly-L-ornithine that was used as starting material.

Compound V is toxic in nanomolar concentrations to two cell lines possessing a large number of EGF receptors. Since the attachment of $hrEGF_L$ to the toxin vehicle caused a 20-fold increase in the in vitro toxicity to A 431 cells, it appears likely

that the hormone had mediated a specific uptake of the toxin conjugate into the cells, probably via receptor-mediated endocytosis. This idea is supported by the finding that in another cell line (THF), which possesses only 5% of the EGF receptors present on A 431 cells, the enhancement of cytotoxicity was 7-fold less. Although the cytotoxicity of compound V decreased in parallel with the number of EGF receptors, the dependence is obviously not linear.

A specific role of $hrEGF_L$ in the uptake of compound V also seems likely when the small changes in physical properties caused by the attachment of the hormone molecules are considered. The amanitin-loaded carrier is peptidic in nature as is the hormone, and also the molecular mass of the toxin complex (approximately 50 kDa) is not grossly changed when one or two small protein molecules (6 kDa) are added. It is difficult, therefore, to imagine that after attachment of the hormone the toxin complex should acquire any unspecific transporting properties that are absent in the unlabeled complex. It is more likely that the hormone molecules had attached the toxin complex to the EGF receptors and thus mediated its internalization. Experiments to suppress the toxic activities of compound V by the addition of excess $hrEGF_L$ were not done because $hrEGF_L$ is rare, and competition of $hrEGF_L$ bound to compound V for the EGF receptor was shown in binding experiments with A 431 cells (Figure 4).

The enhancement of toxicity by a factor of 20 observed for compound V appears modest when compared to the enhancement that has been found for coupling of ricin A to EGF (Cawley & Herschman, 1980). This chimeric protein showed an in vitro toxicity that was orders of magnitude higher than that of unlabeled ricin A. A direct comparison, however, is not appropriate. EGF in combination with ricin A has two functions, first to direct delivery of the toxin to certain cells and second to restore the biological activity of ricin A that is lost when it is separated from its binding protein, ricin B. This second function of the signal protein does not apply to the amanitin–poly-L-ornithine conjugate. Here, the only role of the hormone was to mediate the uptake of the conjugate into cells with appropriate receptors. The comparison between ricin A and amatoxin conjugates is complicated further by the large difference in the intracellular threshold concentrations of the two toxins. While nanomolar concentrations of amanitin are required to inhibit transcription, ricin A prevents translation at concentrations that are orders of magnitude lower.

Similar to most of the protein derivatives of β-amanitin, the conjugates with poly-L-ornithine show an enhanced in vivo toxicity. Depending on their amanitin load, amanitin conjugates with the basic poly(amino acid) were 5–10 times more toxic than free β-amanitin. The enhanced toxicity disappeared when the conjugates were labeled with $hrEGF_L$. Remarkably, compound V was even two times less toxic than free β-amanitin. It seems likely, therefore, that the in vivo application of compound V is only limited by the poly-L-ornithine-specific toxicity that becomes apparent at concentrations of 2 mg of poly-L-ornithine/kg bwt. However, the low in vivo toxicity may reflect the existence of an unknown clearing process in the body of the mouse. Naturally, sequestering of compound V by uptake into tissues bearing EGF receptors would limit the applicability of the conjugates against tumor cells. In the β-amanitin–poly-L-ornithine conjugates not labeled with $hrEGF_L$ both kinds of toxicity, those of amanitin and of poly-L-ornithine, were seen to develop, depending on dosage. A more detailed toxicity study and a histological examination of the target tissues of the various amanitin derivatives are in progress.

One potential application of $hrEGF_L$-labeled amanitin poly-L-ornithine conjugates is with epidermal cancer cells that express a larger number of EGF receptors than normal keratinocytes do. Such overexpression of EGF receptors (2–50-fold) has been reported for certain squamous cell carcinomas (Yamamoto et al., 1986; Gullick et al., 1986), particularly for carcinomas of esophagus and lung (Ozawa et al., 1987, 1988; Ozanne et al., 1985; Sherwin et al., 1981) and for human breast cancer (Ro et al., 1988). The presence of EGF receptors has also been found to be related to the metastatic potential of breast tumors and lymph node deposits (Sainsbury et al., 1985). The presence or increase of EGF receptors in cancer cells has hitherto been used mainly as a diagnostic tool, but it seems worthwhile to consider its therapeutic significance as well. It remains to be proved, however, that in all cases a larger number of EGF receptors on the cell surface correlates with a higher susceptibility of these cells to EGF-mediated toxin conjugates.

Now that protein-labeled poly-L-ornithine-supported amatoxins have been prepared, it will be of interest to bind other tissue hormones to the toxin vehicles, for example, bombesin or LDL, and to test these conjugates for their specific cytotoxic activities. Moreover, ligands other than hormones can be imagined, for example, CD4 protein or certain antigens, which would specifically deliver amatoxin conjugates to unwanted cells of the immune system, such as lymphocytes infected by HIV or involved in autoimmune diseases.

Acknowledgments

We thank Dr. W. W. Franke of the Deutsche Krebsforschungszentrum for providing the cell lines and for helpful discussions and Drs. D. Schmähl and M. Berger of the Deutsche Krebsforschungszentrum for performing the in vivo toxicity assays. The work was supported by a grant from the Deutsche Forschungsgemeinschaft (Fa-126/7-1).

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The Carboxy Terminus of the α Subunit of Tubulin Regulates Its Interaction with Colchicine†

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Received February 14, 1990

ABSTRACT: Controlled proteolysis of goat brain tubulin by subtilisin was carried out to investigate regulatory aspects of the binding of colchicine to tubulin. Tubulin S, obtained by the cleavage of the carboxyl termini of both the α- and β-subunits of tubulin by subtilisin, exhibited the following differences compared to native tubulin: (a) Reaction with colchicine, which has an optimum pH of 6.8, becomes independent of pH (in the range 5.7-8.0). (b) The colchicine-binding site, which is labile at 37 °C ($t_{1/2}$ = 4-5 h), becomes highly stable ($t_{1/2}$ > 12 h). (c) The affinity for colchicine is lowered. (d) This lowering of affinity arises from a faster dissociation (higher off rate) of the complex. The above characteristics of tubulin S were not shown by a partially digested hybrid in which the C-terminus of the β-subunit alone was cleaved. The hybrid behaved very much like the undigested native protein. These results strongly suggest that the regulatory switch for colchicine-tubulin interaction is located in a small region (about 15 residues) of the C-terminus of the α-subunit of tubulin. Possibilities of the C-termini being involved in nonbonded contacts with the main body of tubulin are also noticed from the change in conformation between tubulin and tubulin S.

Limited proteolysis is a powerful tool in the study of the structures and functions of proteins as well as of regulatory mechanisms of proteins in vivo (Jacobson, 1964; Mihalyi, 1978). This classical technique has been applied to tubulin for the identification of domains for the binding of colchicine (Serrano et al., 1984b; Avila et al., 1987), nucleotides (Maccioni & Seeds, 1983), calcium (Serrano et al., 1986) and microtubule-associated proteins (MAPs)[1] (Serrano et al.,

† This work was supported by the Council of Scientific and Industrial Research, Government of India.
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0006-2960/90/0429-6845$02.50/0