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Differential Inhibition of Cultured Cell Types by α-Amanitin-Bovine Serum Albumin Conjugates

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SUMMARY

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A protein conjugate of α-amanitin was prepared by covalently linking an α-amanitin spacer derivative to bovine serum albumin with carbodiimide. This conjugate had an apparent K_I toward calf thymus DNA dependent RNA polymerase (RNA nucleotidyl transferase, E.C. 2.7.7.6) of 69×10^{-9} M compared with an apparent K_I of 1.8×10^{-9} M for free α-amanitin. The conjugate was 5.2, 2.5, and 0.52 fold more effective than free α-amanitin in inhibiting cell proliferation in human amnionic cells (AV_3), chinese hamster ovary cells (CHO), and mouse lymphocytic leukemia cells (EL4), respectively. Measurement of pinocytotic activity by uptake of [^{125}I] bovine serum albumin indicated that AV_3 cells were 3.6 fold more active than CHO cells whereas EL4 cells possessed negligible pinocytotic activity under the conditions studied. While differential susceptibility of the three cell lines to conjugated α-amanitin may be a function of differences in pinocytotic capability, conjugated α-amanitin is clearly more toxic to cultured cells than would be expected from its *in vitro* inhibition of calf thymus RNA polymerase II. These observations suggest the protein carrier of the conjugate may facilitate binding and possibly uptake of the bound amanitin by cells after which the amanitin may be cleaved, resulting in the inhibition of RNA synthesis and cell growth.

INTRODUCTION

The amanitins are a group of bicyclic peptide toxins isolated principally from members of the fungal genus *Amanita* (1). Their toxicity is derived from their ability to bind specifically to eukaryotic DNA dependent RNA polymerase II (2), inhibiting the synthesis of messenger RNA (3). In an attempt to produce an immunogenic derivative of an amanitin, Cessi and Fiume (4) conjugated β-amanitin to rabbit serum albumin by direct linkage of its free carboxyl group to free amino groups on albumin. In comparison to free β-amanitin, these conjugates displayed an increased toxicity *in vivo* for mice. Other conjugates of β-amanitin and bovine serum albumin (BSA),[1] while retaining some of the inhibitory potential for RNA polymerase II of free β-amanitin (5), were found to have increased cytotoxicity for peritoneal macrophages *in vitro* (6). A conjugate of α-amanitin and BSA first prepared by Faulstich and Trischmann (7) also inhibits RNA polymerase II and displays enhanced *in vivo* tox-

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[1] The abbreviations used are BSA, bovine serum albumin; ADBH, α-amanitin-diazobenzoyl-N-N'-BOC-hexamethylenediamine; ADH, α-amanitindiazobenzoyl-N-hexamethylenediamine; AMA-BSA, α-amanitin-bovine serum albumin; [^{3}H]-TdR, [methyl-^{3}H]-thymidine.

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icity. These results suggest the possibility that amanitin-macromolecular conjugates may exert differential toxicity for different cell types as a function of specific recognition of the macromolecular portion of the conjugate followed by uptake of the conjugate. The restricted specificity and high affinity with which amanitins bind to RNA polymerase II and the possibility of additional specificity of uptake imparted by conjugation with macromolecules make amanitin conjugates potentially useful in studies of mRNA synthesis by specific cells and of receptor mediated recognition and uptake of macromolecules.

In order to explore the parameters of the cytotoxicity of amanitin protein conjugates, we have prepared a conjugate of BSA and α-amanitin diazotized to p-aminobenzoylhexamethylenediamine, evaluated its inhibitory potential to calf thymus RNA polymerase II, and examined the cytotoxic potential of the conjugate toward cell lines growing on a solid surface and in suspension. Preliminary reports of portions of this work have been given (8, 9).

MATERIALS AND METHODS

Preparation of α-amanitin-BSA conjugate. AMA-BSA conjugates were synthesized by minor modification of the method of Faulstich and Trischmann (7). All chemicals were of reagent grade. Mono-BOC-1,6-diaminohexane was prepared according to the procedure of Gieger (10). Acylation of mono-BOC-1,6-diaminohexane with 4-nitrobenzoylchloride yielded N-(4-nitrobenzoyl)-N′-BOC-hexamethylenediamine which after reduction to N-(4-aminobenzoyl)-N′-BOC-hexamethylenediamine was diazotized to α-amanitin. The resulting derivative, α-amanitin-diazobenzoyl-N-N′-BOC-hexamethylenediamine (ADBH) was then used for coupling with BSA. The t-BOC group was removed by dissolving 6 mg dry ADBH in 5 ml dry trifluoroacetic acid, swirling for 1 min at room temperature and immediately evaporating to dryness at 40° *in vacuo* to provide an α-amanitin diazo spacer (ADH) with a free amino group. Twenty milligrams BSA (Sigma, crystallized and lyophilized) dissolved in 2 ml distilled water with 200 mg of N-ethyl-N′-(dimethylaminopropyl)-carbodiimide HCl were added to the dried ADH. After 24 hr at room temperature the mixture was applied to a Sephadex G-75 column and eluted with 0.05% NH_4HCO_3. Individual fractions were analyzed for protein (absorbance at 280 nm), diazo moieties (absorbance at 384 nm) and inhibition of calf thymus RNA polymerase II. The conjugate AMA-BSA eluted as a single peak followed by unreacted ADH which was reused. The AMA-BSA peak was pooled, lyophilized and resuspended in distilled water for determination of concentrations of BSA and bound α-amanitin. For use in culture, the α-amanitin and AMA-BSA solutions were sterilized by filtration through a 0.22 μ Millipore filter.

Inhibition of calf thymus RNA polymerase II. Calf thymus RNA polymerase II was purified through DEAE cellulose chromatography according to the method of Kedinger *et al.* (11). Assay of the inhibition of calf thymus RNA polymerase II was performed as previously described by Preston *et al.* (12) using the reaction mixture described by Cochet-Meilhac and Chambon (13).

Inhibition of cultured cells. Human amnionic AV_3 cells, a normal epithelial-like line, and chinese hamster ovary cells (CHO) were maintained as monolayers in Eagles MEM and McCoy's 5a media, respectively. Mouse lymphocytic leukemia EL4 cells, a transformed lymphocyte line that grows in suspension, were maintained in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum (International Scientific Industries), penicillin (250 units/ml), and streptomycin (125 μg/ml). Cells were grown at 37° under a 95% air: 5% CO_2 humidified atmosphere. Prior to each experiment AV_3 and CHO cells were harvested by a 5 min exposure to 0.08% trypsin, resuspended to a density of 0.5 to 1.0×10^5/ml in the appropriate media and 1.0 ml aliquots were added to sterile scintillation vials. The cultures were allowed to establish growth for 12–16 hr. α-Amanitin, AMA-BSA or media were then added as 100 μl additions for triplicate cultures of each group to give the final concentrations as listed in the tables. For determination of

cell numbers AV_3 and CHO cultures were washed 3 times with 2.0 ml cold balanced salt solution (Gey's A without Ca^{2+} or Mg^{2+}) and treated with 1.0 ml of 0.08% trypsin for 5 min. Following detachment of the cells from the glass, 9.0 ml Gey's A solution was added to each vial and the cells were counted directly with an electronic particle counter (Celloscope, Particle Data, Inc.). EL4 cells were grown in suspension in scintillation vials and were counted directly after dilution with Gey's A solution.

Incorporation of [methyl-^{3}H]-thymidine (Schwartz-Mann) was measured using a procedure similar to that of Ball *et al.* (14). Labeled precursors were added as 100 μl additions in the appropriate medium to give a final concentration of 1 μCi/ml. After 1 hr incubation, incorporation was stopped by one addition of 10 ml ice cold Gey's A solution followed by 3 washes of 5.0 ml of 1.5% perchloric acid. Cultures were then washed once with 95% ethanol and drained. Five percent perchloric acid (1.0 ml per vial) was added and the vials were heated at 80° for 40 min to hydrolyze the nucleic acids. After cooling, 10 ml of a Triton-X-100 based scintillation cocktail were added to each vial for counting in a Beckman LS-133 scintillation counter.

Measurement of pinocytosis. Measurement of pinocytosis was performed by modification of the method of Steinman *et al.* (15). BSA was iodinated with ^{125}I (New England Nuclear, carrier free sodium salt) to a specific activity of 1 μCi/μg with solid state lactoperoxidase (16). For determination of pinocytotic uptake of [^{125}I]-BSA, AV_3 and CHO cultures were grown to confluent monolayers in scintillation vials. To each culture was added 50 μl of [^{125}I]-BSA such that each received approximately 10^7 cpm. After 24 hr the cultures were processed by removal of the medium followed by 6 washes with 2.0 ml of serum free medium. Trypsin (0.5 ml of a 0.08% solution) was added to each and after detachment the cells were removed to a plastic tube for counting. The culture vial was rinsed twice with 1.0 ml medium which was added to the cells for counting. The cells were centrifuged for 10 min at 1500 rpm and the supernate and cell pellets were counted separately. In all cases, negligible activity was found in the supernate. Duplicate cultures which had not received [^{125}I]-BSA were given 50 μl of [^{125}I]-BSA 5 min prior to harvesting, incubated for 5 min and processed as above. The activity remaining in these cultures was subtracted from the 24 hr cultures as a control for nonspecific surface absorption. Pinocytosis by EL4 cells was determined as above except that each wash of serum free medium required a centrifugation step to pellet the cells. After 6 washes, negligible activity remained in the supernate and 5 min background absorption was subtracted from the 24 hr values.

RESULTS

Following purification of the AMA-BSA by Sephadex G-75 chromatography, the molar concentration of bound α-amanitin per mole of BSA was determined from the characteristic absorption spectra presented in Fig. 1. The concentration of α-amanitin was estimated from the molar extinction coefficient (ϵ^{384} = 14000 M^{-1} cm^{-1}) of the azo dye moiety of the spacer molecule (7), since the ADH intermediate contains a 1:1 ratio of azo linkage and α-amanitin. The concentration of BSA was determined from its absorption at 280 nm (ϵ^{280} = 40000 M^{-1} cm^{-1}) after correcting for the absorption of the azo compound at 280 nm. For all experiments reported here, the molar ratio of α-amanitin to BSA of the AMA-BSA conjugate was 1.2.

The effect of conjugation on the interaction of α-amanitin with calf thymus RNA polymerase II *in vitro* can be seen in Fig. 2. Analysis of the kinetics of inhibition by use of a Dixon plot (17) of the reciprocal of velocity versus concentration of inhibitor for three different substrate concentrations yields apparent inhibition constants (K_I) of 1.8×10^{-9} M for free α-amanitin, 3.0×10^{-9} M for the ADH intermediate and 69.0×10^{-9} M for the AMA-BSA conjugate. For free α-amanitin and the ADH intermediate the inhibition is non-competitive with respect to substrate concentration. This agrees well with the inhibition constant and non-competitive inhibition obtained by Chochet-Meilhac and Chambon for α-amanitin (13).

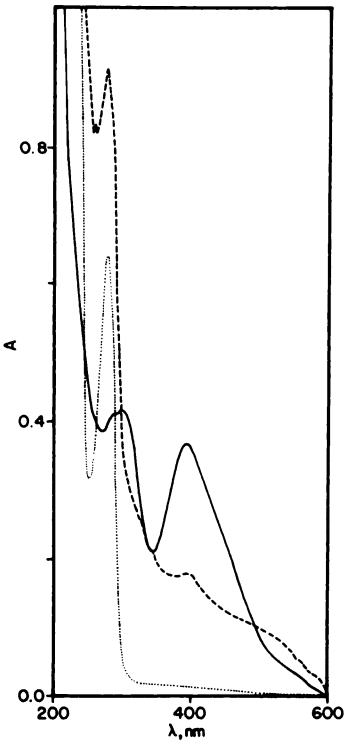

FIG. 1. *Absorption spectra of the α-amanitin spacer derivative and the α-amanitin bovine serum albumin conjugate*

Absorption spectra of ADBH (solid line), BSA (dotted line), and AMA-BSA (dashed line) were determined with a 10 mm light path with a Beckman Model 24 spectrophotometer at room temperature. ADBH was dissolved in methanol (33 μg/ml); BSA and AMA-BSA were dissolved in 0.05% NH_4HCO_3, pH 8.0 to concentrations of 1.0 mg/ml each.

The similarity of the apparent K_I for both α-amanitin and ADH indicates that the modification of α-amanitin by diazotization to provide the ADH intermediate did not significantly alter the binding affinity of α-amanitin for calf thymus RNA polymerase II. For the AMA-BSA conjugate, an average of the three abscissa intercepts yields an apparent inhibition constant much higher than that for unbound α-amanitin, indicating that coupling to BSA results in a 38-fold decrease in the binding affinity for calf thymus RNA polymerase II. This decrease in binding affinity may be a result of steric hindrance by the BSA portion of the conjugate rather than a conformational alteration of α-amanitin by the spacer moiety. Furthermore, the lack of convergence of the three lines obtained with different UTP concentrations implies that conjugation to BSA not only decreases binding affinity but alters the nature of the inhibition from the strictly non-competitive type.

The effect of the AMA-BSA conjugate and free α-amanitin on cultured mammalian cells was examined with CHO, EL4 and AV_3 cell lines. In all experiments reported the cells were allowed to establish growth for 12–16 hr. Varying concentrations of the inhibitors were then added and 48 hr later the cells were analyzed either for inhibition of cellular proliferation or for inhibition of incorporation of [^{3}H]-TdR into an acid insoluble product.

Table 1 presents the effects of equivalent molar concentrations of free versus conjugated α-amanitin on cell proliferation of CHO and EL4 cells. For inhibition of cell growth, conjugated α-amanitin is slightly more effective an inhibitor of CHO cells than free α-amanitin while for the EL4 cells, the reverse was observed. For CHO cultures, concentrations of α-amanitin of 5.45×10^{-7} M and 10.90×10^{-7} M decreased the [^{3}H]-thymidine incorporation from 2.75×10^4 cpm to 1.36×10^4 cpm (51% inhibition) and 0.75×10^4 cpm (73% inhibition), respectively. Equivalent concentrations of conjugated amanitin decreased the incorporation from 2.62×10^4 cpm to 1.77×10^4 cpm (33% inhibition) and 1.43×10^4 cpm (46% inhibition). In contrast to the inhibition of cell proliferation, the apparent inhibition of DNA synthesis in CHO cells by free amanitin was slightly greater than that found with conjugated amanitin. However, the magnitudes of the differences of inhibition comparing one assay to the other were not great, indicating that CHO cells are nearly as sensitive to free α-amanitin as to that conjugated to BSA. Under the conditions of these assays, EL4 and AV_3 cells did not incorporate sufficient quantities of [^{3}H]-thymidine to permit a quantitative evaluation of the inhibitors on DNA synthesis in these cell lines.

As shown in Table 2, AV_3 cells are slightly more sensitive than CHO cells in inhibition by free α-amanitin. However, at

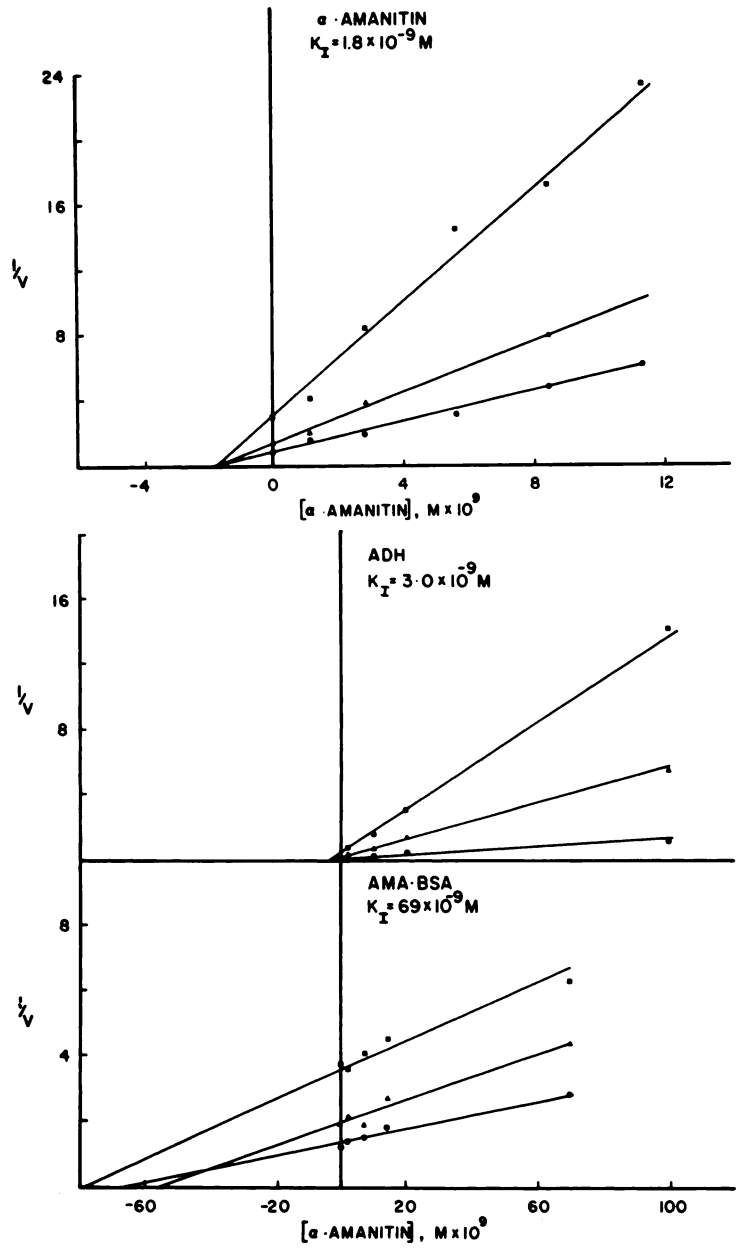

FIG. 2. *Inhibition of calf thymus RNA polymerase II by α-amanitin, ADH, and AMA-BSA*

Reactions were run for 10 min at 37° as described in MATERIALS AND METHODS at three different UTP concentrations: 0.004 mM (■), 0.008 mM (▲), and 0.016 mM (●). The best fit for each curve was established by the method of least squares. For ADH and AMA-BSA, the concentrations of α-amanitin were determined from the extinction coefficient for the diazo linkage.

2×10^{-7} M concentration of conjugated α-amanitin, 50% inhibition of AV_3 cells is seen whereas a much greater amount of free α-amanitin (estimated as 11×10^{-7} M by interpolation) is required for the equivalent inhibition. This increased susceptibility to conjugated α-amanitin implies a preferential uptake of AMA-BSA and/or modification of the conjugate to a more toxic derivative by the AV_3 cells.

TABLE 1

Cytotoxicity of α-amanitin and AMA-BSA to chinese hamster ovary and mouse lymphocytic leukemia cells

CHO and EL4 cells were exposed to increasing concentrations of α-amanitin and AMA-BSA and measured with respect to cell proliferation as described in MATERIALS AND METHODS. Molarity for AMA-BSA refers to the concentration of α-amanitin bound.

Inhibition	α-Amanitin	CHO cells		EL4 cells	
		Total cells × 10^{-5}	Percent inhibition	Total cells × 10^{-5}	Percent inhibition
	(M × 10^7)				
None	0	2.90	0	3.65	0
α-Amanitin	2.72	2.74	5	3.31	9
α-Amanitin	10.9	2.26	22	2.83	22
α-Amanitin	27.2	0.78	74	1.74	52
α-Amanitin	54.5	0.27	91	0.53	85
None	0	3.13	0	3.65	0
AMA-BSA	2.72	1.91	39	3.41	6
AMA-BSA	10.9	1.32	58	3.38	7
AMA-BSA	27.2	0.802	74	2.63	28
AMA-BSA	54.5	—		1.68	54

TABLE 2

Cytotoxicity of α-amanitin and AMA-BSA to human amnion AV_3 cells

AV_3 cells were exposed to increasing concentrations of α-amanitin and AMA-BSA and measured with respect to cell proliferation as described in MATERIALS AND METHODS. Molarity for AMA-BSA refers to the concentrations of α-amanitin bound.

Inhibitor	Amanitin	Total cells × 10^{-4}	Percent inhibition
	(M × 10^7)		
None	0	6.4	0
α-Amanitin	1.10	6.0	6
α-Amanitin	2.20	5.5	14
α-Amanitin	4.40	5.1	20
α-Amanitin	8.80	3.5	45
α-Amanitin	17.60	1.9	70
None	0	8.0	0
AMA-BSA	0.56	7.1	12
AMA-BSA	1.10	4.5	44
AMA-BSA	2.20	3.8	53

Cell size distribution analysis after 48 hr in culture with free or conjugated α-amanitin (Fig. 3) demonstrates no shift in cell size that might have resulted from a growing but nondividing population of cells or from a population that was dividing in the absence of DNA synthesis. Thus, both α-amanitin and AMA-BSA cause a simultaneous arrest in cell growth and division in all three lines. Furthermore, correlation of inhibition of cellular proliferation with cell viability based on trypan blue exclusion indicated that AMA-BSA was clearly cidal.

Since differential uptake of conjugated α-amanitin could occur by means of specific receptors or by increased non-specific pinocytosis, we measured the pincytotic capabilities of the three cell lines by use of ^{125}I labeled BSA. The BSA was labeled to a high specific activity (1 μCi/μg) by use of lactoperoxidase coupled to Sepharose 4B in order to minimize any conformational changes in the BSA. The uptake of labeled BSA was determined after 24 hr in culture. Table 3 presents a summary of this information compared with the molar concentration of free and conjugated α-amanitin required for 50% inhibition of cell growth for all three cell lines. AV_3 cells are approximately 3.5 times more active in the pinocytotic uptake of labeled BSA than are CHO cells. EL4 cells, under the same conditions, took up negligible amounts of labeled BSA. A correlation between enhanced susceptibility to conjugated α-amanitin and increased pinocytotic uptake of iodinated BSA is seen for AV_3 cells and to a lesser extent, for CHO and EL4 cells. The greater the pinocytotic capability, the more susceptible the cell is to conjugated α-amanitin.

DISCUSSION

Investigations of macromolecular derivatives of β-amanitin demonstrated that conjugated β-amanitin was more toxic *in vivo* for rats and mice (4, 18, 19) even

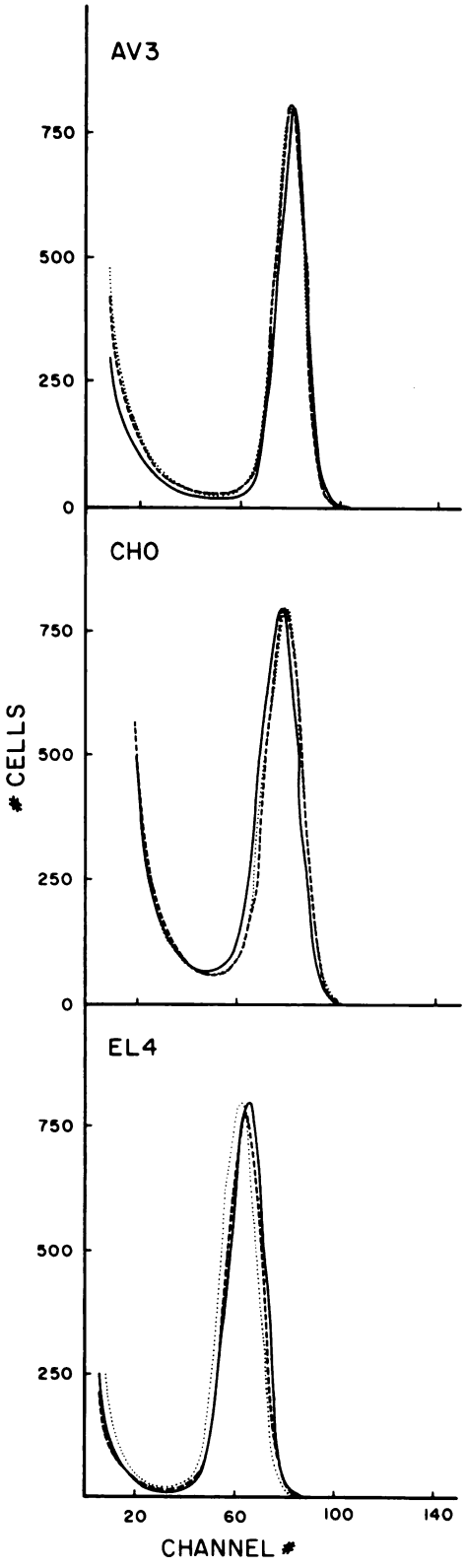

TABLE 3

Comparison of cytotoxicity of α-amanitin and AMA-BSA with pinocytotic activities of AV$_3$, CHO, and EL4 cells

Concentrations (M × 10^7) of α-amanitin and AMA-BSA which gave 50% inhibition of cell proliferation were interpolated from dose response data presented in Tables 1 and 2 and are presented as ID_{50} values. Details for determining pinocytotic uptake of [^{125}I]BSA are given in MATERIALS AND METHODS.

Cell Type	ID_{50}		ID_{50} ratio α-amanitin/ AMA-BSA	Pinocytosis of [^{125}I]-BSA
	α-Amanitin	AMA-BSA		
	(M × 10^7)			(μg/cell × 10^{10})
AV$_3$	11	2.1	5.2	2.83
CHO	18	7.3	2.5	0.78
EL$_4$	26	50	0.52	<0.2

though it inhibited RNA polymerase II less effectively than free β-amanitin (5). Histological examinations of animals treated with conjugated β-amanitin revealed that cells believed to have high rates of protein uptake, e.g., kidney proximal tubule cells and liver sinusoidal cells, in addition to peritoneal macrophages *in vitro*, were preferentially damaged by conjugates (20). In this study we wished to quantitatively examine the effects of α-amanitin linked with diazobenzoyl-hexamethylene spacer moieties on cells not noted for high rates of nonspecific protein uptake. This would enable us to then explore the feasibility of selectively inhibiting certain cultured cells by their response in receptor mediated recognition of the macromolecular portion of various α-amanitin conjugates.

The shift in absorption peaks for both BSA and ADH following conjugation and the coelution of protein and calf thymus RNA polymerase II inhibiting activity from a Sephadex G-75 column is indicative of covalent linkage. In spite of the greatly reduced affinity of the conjugate for calf

FIG. 3. *Size distributions of AV$_3$, CHO, and EL4 cells exposed to α-amanitin and AMA-BSA*

Details for suspending cells and exposure to inhibitors are described in MATERIALS AND METHODS. A total of 110 channels were scanned in a Celloscope particle counter. Untreated control cultures are noted by the solid line. Cells exposed to α-amanitin and AMA-BSA are noted by the dashed and dotted lines, respectively.

thymus RNA polymerase II, AMA-BSA was more toxic for AV_3 cells and approximately equally toxic for CHO and EL4 cells in comparison with free α-amanitin on a molar basis. This would imply a preferential uptake of conjugated α-amanitin, modification of the conjugate to a more toxic derivative or some combination of the two occurs in susceptible cells. The extent of uptake of [^{125}I]-BSA by AV_3 cells correlates with its increased susceptibility to AMA-BSA, indicating that pinocytosis is a major contributing factor in the cytotoxicity of α-amanitin conjugates. The relative sensitivity of macrophages to inhibition by conjugates of β-amanitin and bovine serum albumin has already been demonstrated (21) and indentifies the important role played by phagocytosis and/or pinocytosis in the toxicity of amanitin-protein conjugates to mammalian cells.

The fact that CHO and EL4 cells are almost as susceptible to AMA-BSA as they are to free α-amanitin in spite of their reduced pinocytotic activity, however, indicates that increased pinocytotic uptake of AMA-BSA relative to free α-amanitin alone cannot account for the observed toxicity of the conjugate. Uptake of conjugate via specific receptors for some portion of the conjugate, and/or lysozomal digestion of internalized AMA-BSA followed by release of free α-amanitin most probably contribute to the toxicity of AMA-BSA conjugates. The lysosomal cleavage of amanitin from a conjugate with bovine serum albumin has been suggested by Fiume and Barbanti-Brodano to occur after these conjugates have penetrated the cell (21).

The use of several different azo-spacer derivatives of α-amanitin for coupling to macromolecules will facilitate investigation of the mechanism of enhanced toxicity seen with AMA-BSA conjugates. Variations in spacer chain length and mode of coupling, i.e., via free amino versus carboxyl groups, as well as in the protein itself should yield other useful derivatives of α-amanitin with cell specific cytotoxic properties. Further investigations of the nature of the interaction of α-amanitin conjugates with specific cell receptors are currently underway with conjugates for which defined membrane receptors exist.

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