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Asialoglycoprotein receptor mediates the toxic effects of an asialofetuin-diphtheria toxin fragment A conjugate on cultured rat hepatocytes

(disulfide-linked hybrid protein/protein synthesis/lectin/cell-type-specific killing)

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ABSTRACT **We have constructed a toxic hybrid protein that is recognized by asialoglycoprotein (ASGP) receptors of cultured rat hepatocytes. The conjugate consists of fragment A of diphtheria toxin (DTA) linked by a disulfide bond to asialofetuin (ASF). This conjugate is highly toxic, inhibiting protein synthesis in primary rat hepatocytes at concentrations as low as 10 pM. The ASF-DTA conjugate was 600 and 1800 times as toxic as diphtheria toxin and DTA, respectively, on primary rat hepatocytes. The ASGP receptor recognizes galactose-terminated proteins. We tested a series of glycoproteins for their ability to block the action of the ASF-DTA conjugate. Fetuin and orosomucoid, two glycoproteins with terminal sialic acid on their oligosaccharide chains, did not block the action of the conjugate. Their galactose-terminated asialo derivatives, ASF and asialoorosomucoid, as expected, did block the action of the conjugate. The *N*-acetylglucosaminyl-terminated derivative (asialoagalactoorosomucoid) had no appreciable effect on the activity of the conjugate. We tested the ASF-DTA conjugate on six cell types; except for primary rat hepatocytes, none of them were affected by a high concentration (10 nM) of ASF-DTA conjugate. A fetuin-DTA conjugate was less toxic by a factor of 300 than the ASF-DTA conjugate and exerted its effects primarily through non-receptor-mediated mechanisms. The highly toxic ASF-DTA conjugate is cell-type specific, and its action is mediated by a well-characterized receptor, whose mechanism of receptor–ligand internalization has been extensively investigated.**

The successful construction of disulfide-linked protein conjugates, composed of a binding protein (e.g., a lectin, hormone, or antibody) and the enzymatic subunit of a toxin [e.g., fragment A of diphtheria toxin (DTA) or the ricin A chain], demonstrates the feasibility of preparing receptor-specific toxic agents. However, only plant lectin (1, 2) and antibody (3, 4) conjugates of DTA have proved to be appreciably toxic. Because receptors for specific plant lectins are present on many types of mammalian cells, the lectin conjugates would not be expected to show cell-type specificity. The nature of the receptors for the toxic antibody conjugates are not known (3, 4).

DTA conjugates also have been constructed with hormones as ligands. In these cases the physiological significance of the receptors and the cell-type specificity for the conjugates is clear. However, an insulin-DTA conjugate was toxic only at high concentrations (0.1 μM) (5). DTA conjugates of human placental lactogen (6) and epidermal growth factor (7) were not toxic, despite the integrity of the binding and enzymic activities of the respective proteins in those conjugates. The availability of highly toxic hybrid proteins capable of recognizing specific cell-surface receptors could prove to be valuable, as has been suggested, in selecting cells with altered receptor functions (5, 8), as tumoricidal agents (3, 4, 9, 10), and in the study of the transport of protein toxins into cells (6, 9, 11, 12).

Ashwell, Morell, and coworkers initially described (13), purified, and characterized (14, 15) a hepatocyte cell-surface receptor in mammals, the asialoglycoprotein (ASGP) receptor, which recognizes desialyated (galactose-terminated) glycoproteins, such as asialofetuin (ASF) and asialoorosomucoid (16–18), and mediates their internalization (19–25). A receptor of similar function but which specifically recognizes *N*-acetylglucosaminyl-terminated glycoproteins, is present in avian (chicken) liver (26, 27). Details of the binding (18, 20, 24), internalization (20, 24), and degradation (20, 24) of ASGPs have been established using cultured rat hepatocytes. Although both the ASGP receptor and the bound desialyated glycoprotein appear to be internalized, the receptor is apparently recycled, whereas the ligand is degraded (21, 24).

Because the ASGP receptor molecule and its internalization mechanism are well-characterized, we chose it as a model system for the construction of a cell-type- and receptor-specific toxic conjugate. Previous studies have demonstrated that modified ASGPs can be used to direct specific agents to hepatocytes through the ASGP receptor (28, 29). We endeavored to construct a toxic ASGP conjugate using only the enzymatic polypeptide chain of a protein toxin. Diphtheria toxin (30, 31) is composed of two functional moieties: (*i*) a cell-surface binding protein (fragment B) that has no enzyme activity and is nontoxic by itself and (*ii*) an enzyme (DTA) that irreversibly inhibits (under physiological conditions) protein synthesis in eukaryotic cells by catalyzing the transfer of the ADP-ribose moiety of NAD to elongation factor 2, destroying the latter's activity. Although diphtheria toxin is secreted as a single polypeptide chain by *Corynebacterium diphtheriae*, the toxin is highly susceptible to limited proteolytic cleavages that leave the A and B fragments joined by a single disulfide bridge. DTA, which requires the B fragment to intoxicate cells, has a single sulfhydryl group available for forming disulfide-linked conjugates.

We report here the successful construction of an ASF-DTA conjugate. This conjugate is highly toxic to cultured hepatocytes and is ASGP receptor specific.

METHODS

Protein Purification. DTA, gift of D. G. Gilliland and R. J. Collier (University of California, Los Angeles), was prepared

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Abbreviations: ASGP, asialoglycoprotein; ASF, asialofetuin; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; ASF-PDP and F-PDP, 2-pyridyldithiopropionyl derivatives of asialofetuin and fetuin, respectively; DTA, fragment A of diphtheria toxin; ASF-DTA and F-DTA, hybrid proteins consisting of fragment A of diphtheria toxin coupled to asialofetuin or fetuin, respectively; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; ED_{50}, amount of agent required to give 50% inhibition of protein synthesis in cells after 24 hr.

from diphtheria toxin (Connaught Laboratories, Willowdale, Ontario, Canada) as described (32) and was heated to 80°C for 10 min to inactivate any traces of toxin.

Glycoprotein and Polysaccharide Preparations. Fetuin (Sigma) was desialyated enzymatically with agarose-bound *Clostridium perfringens* neuraminidase (Sigma type IX). Protein (300 mg) was incubated overnight at 37°C with gentle shaking in 0.2 M sodium acetate (pH 5.0) containing 0.02% azide and one unit of enzyme (one unit liberates 1 μmol of *N*-acetylneuraminic acid from *N*-acetylneuraminic acid-lactose per min at pH 5.0, 37°C). ASF was recovered by chromatography on Sephadex G-25, and sialic acid content was determined by using the thiobarbiturate assay (33). A minimum of 80% of the sialic acid was removed. Orosomucoid was a gift from M. Wickerhauser (American National Red Cross Blood Research Laboratory). Asialoorosomucoid was a gift of J. Whitehead (Gastrointestinal Research Unit of the Veteran's Administration Hospital, San Francisco). Asialoagalactoorosomucoid and phosphomannan from the yeast *Hansenula holstii* NRRL Y-2448 were from J. Paulson and L. Rome, respectively (University of California, Los Angeles). Baker's yeast mannan was from Sigma.

Reaction of ASF and Fetuin with *N*-Succinimidyl 3-(2-Pyridyldithio)propionate (SPDP). Fetuin and ASF were derivatized on primary amino groups with SPDP (Pharmacia) by incubating them with 2.5 molar equivalents of reagent for 30 min at room temperature in 0.2 M sodium phosphate (pH 7.4). The derivatives containing 2-pyridyldithiopropionate (PDP) groups (ASF-PDP and F-PDP) were recovered by Sephadex G-25 chromatography. The extent of derivatization was determined as described (34). An aliquot of protein was reduced with dithiothreitol (final concentration, 5 mM), and the amount of pyridine-2-thione released was measured by using the extinction coefficient at 343 nm ($8.08 \times 10^3\ M^{-1}cm^{-1}$). The ASF-PDP and F-PDP derivatives contained an average of 1 mol of reagent per mol of protein.

Preparation of F-DTA and ASF-DTA Conjugates. Prior to conjugation with ASF-PDP and F-PDP, DTA was reduced in 0.5 M Tris·HCl/0.1 mM EDTA/0.1 M dithiothreitol, pH 8.0, for 4 hr at room temperature. The reduced protein was desalted on G-25 Sephadex equilibrated in 20 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes), pH 7.4/0.02% sodium azide. For conjugation, 1 mg (50 nmol) of reduced DTA was added to 2.5 mg (55 nmol) of ASF-PDP or F-PDP in a total vol of 5 ml of 20 mM Tes. The mixtures were then dialyzed twice against 50 vol of 20 mM Tes (pH 8.0). After dialysis for 36 hr at 4°C, the mixtures were concentrated by ultrafiltration to 1.5 ml. The conjugates were then purified by chromatography on a 1.5 × 45 cm Sephadex G-100 (Superfine) column equilibrated at 4°C with 20 mM sodium phosphate, pH 6.8/0.1 M NaCl/0.02% sodium azide.

Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed as described (35). Gels were processed through fixing, staining, and destaining as described (36).

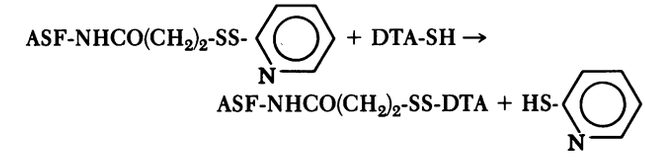
Protein Determination. The protein concentrations of solutions of fetuin, ASF, and DTA were determined by using extinction coefficients (1 mg/ml) at 280 nm of 0.45, 0.45, and 1.5, respectively. An extinction coefficient for the ASF-DTA and F-DTA conjugates was determined by assuming a weighted contribution of the proteins in the conjugates. This value at 280 nm was 0.77 for a 1 mg/ml solution of either conjugate.

Cell Culture. Rat hepatocytes were isolated by using a collagenase perfusion method as described (37). The cells were seeded into 17-mm multiwell tissue culture dishes (24 wells; Costar) in Dulbecco's modified Eagle's medium (GIBCO 78-5433) supplemented per liter with 84 mg of L-ornithine, 584 mg of L-glutamine, 4.5 g of glucose, 2.02 g of sodium bicarbonate, 2.38 g of Hepes, and 20% (vol/vol) heat inactivated (45 min at 56°C) fetal bovine serum. Only those cultures that showed greater than 95% viability (trypan blue exclusion) upon plating were used in experiments. The cells were seeded in 0.5-ml vol at 3.3×10^5 cells per ml. After 4–5 hr, 65–75% of the cells adhered to the dishes. The medium was then removed, and experimental agents were introduced in fresh medium containing no serum. Primary cultures of bovine aortic endothelial cells were prepared by collagenase treatment (38) and were provided by Judith Berliner (University of California, Los Angeles). There were $\approx 2 \times 10^5$ cells per well. Primary cultures of rat heart cells were prepared as described (39) and were provided by E. Sato and I. Harary (University of California, Los Angeles). There were $\approx 6 \times 10^4$ cells per well. Mouse 3T3 cells and green monkey kidney (Vero) cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Chinese hamster ovary cells (CHO-Kl, obtained with the Vero cells from R. Collier) were maintained in Ham's F-12 nutrient medium containing 5% fetal bovine serum.

Protein Synthesis. Two assays were used. In both assays, agents were added to the cells in 1 ml of appropriate culture medium, the cells were incubated for 24 hr at 37°C in the presence of the agents, the medium was removed, and the cells were labeled for 2 hr with [^{3}H]leucine. All cells received 0.5 ml of minimal Eagle's medium containing Earle's salts (lacking leucine), 10 mM Hepes (pH 7.4), 10% dialyzed serum (established cell strains only), and either 1 μCi (established cell strains) or 5 μCi (primary cells) of [^{3}H]leucine (1 Ci = 3.7×10^{10} becquerels). Primary cells were maintained in 17-mm, 24-well tissue culture dishes. After the labeling procedure, the primary cells were solubilized in 0.1 M KOH, the protein was precipitated by adding 10% trichloroacetic acid (final concentration), and the samples were collected on Whatman GF/C filters and processed for scintillation counting as described (40). The established cell strains were seeded at 8×10^4 cells per ml (1 ml) into borosilicate scintillation vials, allowed to incubate 24 hr prior to the addition of toxins, and assayed as described for protein synthesis (7, 41). Primary cells incorporated 18,000–25,000 cpm of [^{3}H]leucine, whereas the established cell strains incorporated 20,000–40,000 cpm of tritium. Assays were performed in duplicate or triplicate.

RESULTS

Preparation and Purification of ASF-DTA and F-DTA Conjugates. Primary amino groups of ASF and fetuin were derivatized with SPDP so that ASF-PDP and F-PDP derivatives contained an average of one mol of reagent per mol of protein. The ASF-DTA and F-DTA conjugates were formed by mixing the ASF-PDP and F-PDP derivatives with reduced DTA and allowing thiol–disulfide interchange to occur:



The conjugations were efficient when the PDP derivatives were added in excess over DTA. Gel filtration on Sephadex G-100 (data not shown) removed unconjugated DTA, ASF-PDP, or F-PDP from the reaction mixtures.

ASF-PDP, DTA, and the ASF-DTA conjugate were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1). Slots 1–3 contained unreduced protein samples. ASF-PDP (slot 1) contained two major bands of $M_r \approx 45{,}000$. The unreduced DTA preparation (slot 2) contained three bands: DTA dimer (M_r, 42,000), DTA monomer (M_r, 21,000), and a proteo-

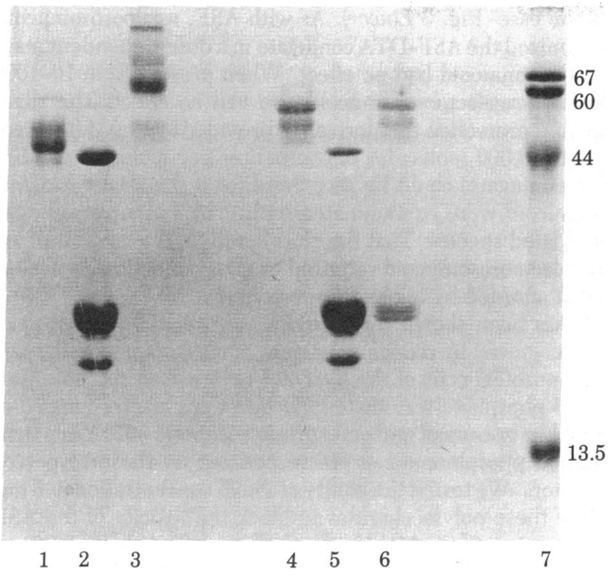


FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of the ASF-DTA conjugate. Proteins were denatured in 1% NaDodSO$_4$/10% (vol/vol) glycerol/0.2 mM EDTA/50 mM Tris·HCl, pH 6.8/bromphenol blue (tracking dye). After an incubation of 15 min at 37°C, samples of the denatured but unreduced proteins were applied to slots 1–3 of the gel. Dithiothreitol was added to the remainder of the samples (final concentration, 50 mM), the contents of the tubes were mixed, and samples were immediately applied to slots 4–6. Electrophoresis was performed with 12.5% (wt/vol) polyacrylamide gel. Slots: 1 and 4, ASF-PDP (3.6 μg); 2 and 5, DTA (7.7 μg); 3 and 6, ASF-DTA conjugate (3.6 μg); 7, M_r markers shown $\times 10^{-3}$ (bovine serum albumin, 67,000; catalase, 60,000; ovalbumin, 44,000; cytochrome *c*, 13,000).

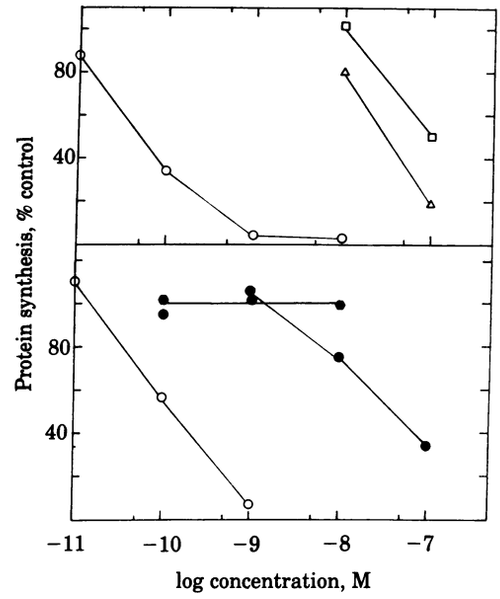


FIG. 2. Sensitivity of primary rat hepatocytes to the ASF-DTA conjugate. The incubation with toxins and subsequent assay for protein synthesis were as described. (*Upper*) ASF-DTA conjugate (○), diphtheria toxin (△), and DTA (□). (*Lower*) In a separate experiment: ASF-DTA conjugate (○), unconjugated ASF and DTA in equimolar amounts (◐), and F-DTA conjugate (●).

lytic fragment of DTA (M_r, 17,000). The prominent band in the ASF-DTA conjugate preparation (slot 3) had a M_r of 66,000, as would be expected from the size of its constituent polypeptides. The preparation may have contained a small amount of higher order conjugates (more than one DTA molecule per mole ASF). Slots 4–6 contained proteins that were exposed to a brief reduction with 50 mM dithiothreitol prior to electrophoresis. Reduced ASF-PDP (slot 4) migrated more slowly than the unreduced protein (slot 1); this altered migration also occurred with unmodified ASF (data not shown). Glycoproteins often migrate anomalously in NaDodSO$_4$ gels (42). The brief treatment with dithiothreitol was not sufficient to dissociate all of the DTA dimer (slot 5); however, treatment of this same preparation for 1 hr at 37°C dissociated all of the DTA dimer (data not shown). The brief reduction completely cleaved the ASF-DTA conjugate (slot 6) into ASF (slot 4) and DTA (slot 5). The proteolytic fragment of DTA was not incorporated into the conjugate. The fetuin conjugate was of a purity similar to the ASF-DTA conjugate, and could also be readily cleaved into its constituent chains by dithiothreitol (data not shown).

Toxicity of the ASF-DTA and F-DTA Conjugates on Primary Rat Hepatocytes. We used the criterion of a 50% inhibitory concentration (ED_{50}) after exposure to toxin for 24 hr to compare the relative abilities of several agents to inhibit protein synthesis in rat hepatocytes. Cells derived from rats and mice are known to be relatively insensitive to diphtheria toxin (2, 31), probably because they lack an appropriate toxin uptake system. In agreement with that observation, we found that neither DTA (ED_{50} of 100 nM) or diphtheria toxin (ED_{50} of 30 nM) was appreciably toxic to the hepatocytes (Fig. 2 *Upper*). For comparison, the typical ED_{50} values for diphtheria toxin on susceptible cell lines were 0.1–100 pM (see below and Table 1). The ED_{50} for the ASF-DTA conjugate was 50 pM; thus, it was 600 and 1800 times as toxic as diphtheria toxin and DTA, respectively. Reduction of DTA did not alter its activity; ASF-PDP (10 μM) was not toxic (data not shown).

In a separate experiment we compared the toxicity of the ASF-DTA conjugate both with the F-DTA conjugate and with a mixture of unconjugated ASF and DTA (Fig. 2 *Lower*). In this experiment the ASF-DTA conjugate had an ED_{50} of 0.13 nM, whereas the F-DTA conjugate was less toxic by a factor of 300 (ED_{50} of 40 nM). ASF and DTA added in equimolar quantities in concentrations as high as 10 nM had no inhibitory effect.

The F-DTA conjugate inhibited protein synthesis only at relatively high concentrations, similar to those at which diphtheria toxin was active on the rat hepatocytes. The activity of F-DTA conjugate might be due either to a trace of partially desialyated fetuin in the fetuin preparation used to make the F-DTA conjugate (less than 0.5% would suffice) or to non-receptor-mediated uptake. To pursue this point we examined the effects of fetuin and ASF on the toxicity of the F-DTA conjugate (data not shown). At a concentration of 0.1 μM, the F-DTA conjugate reduced protein synthesis to 23% of control. ASF added at 0.1 μM increased that value to 35%; increasing the concentration of ASF to 10 μM did not provide a significant increase over that

Table 1. Inhibition of protein synthesis in various cell types by ASF-DTA conjugate and diphtheria toxin

Cell type	ED_{50}, pM	
	Diphtheria toxin	ASF-DTA conjugate
Vero	<10	>10,000 (NI)
3T3	>10,000	>10,000 (NI)
CHO	3.5	>10,000 (NI)
Primary rat hepatocytes	30,000	50
Primary rat heart	>100 (NI)	>10,000 (NI)
Primary bovine aortic endothelium	80	>10,000 (NI)

Cells were exposed to a series of concentrations of diphtheria toxin or ASF-DTA conjugate ranging from 1–10,000 pM. The highest concentration of diphtheria toxin tested on rat heart cells was 100 pM. NI, no inhibition at this concentration.

value. Fetuin had no effect on the F-DTA conjugate when present at 0.1 or 1 μM. At 10 μM, fetuin increased the protein synthesis from 23% (without competitor) to 35%. Thus 0.1 μM ASF was as effective as 10 μM fetuin in blocking the activity of the F-DTA conjugate. The protection against the F-DTA conjugate by competitors was thus very modest and could not be increased, at least in the case of ASF (10 μM fetuin was the highest concentration we tested), by increasing the concentration of the competitor. This suggests that the toxicity we observed with the F-DTA conjugate is partially the result of a trace contamination with partially desialyated fetuin (and, therefore, with a trace of the corresponding DTA conjugate) but is primarily the result of a non-receptor-mediated process.

Receptor Specificity of the ASF-DTA Conjugate. We tested a number of potential antagonists of the ASF-DTA conjugate, including glycoproteins, mannan, and phosphomannan. The primary rat hepatocytes were exposed to 1 nM ASF-DTA conjugate in culture medium alone or in medium containing the various inhibitors. In all experiments, the ASF-DTA conjugate inhibited protein synthesis about 95% at that concentration (Fig. 3). At concentrations of fetuin as high as 10 μM, there was no protection against the toxic activity of the conjugate (Fig. 3 *Upper*). In contrast, ASF blocked the action of the conjugate in a dose-dependent fashion (Fig. 3 *Upper*). A concentration of 1 μM ASF completely blocked the toxic activity of the ASF-DTA conjugate.

We then performed a series of experiments with three derivatives of another glycoprotein, orosomucoid. Orosomucoid's oligosaccharide chains, like those of fetuin, contain terminal sialic acid (43). The oligosaccharides of orosomucoid have the following structure in common: sialic acid-α-(2 $\rightarrow$?)-galactose-β(1 $\rightarrow$ 4)-*N*-acetylglucosamine-R, where R is an asparagine-linked core oligosaccharide (44). Desialyation provides a galactose-terminated protein (asialoorosomucoid). The terminal galactose residues of asialoorosomucoid can be removed enzymically, generating an *N*-acetylglucosaminyl-terminated protein (asialoagalactoorosomucoid) (45). Because the ASGP receptor recognizes galactose-terminated proteins, only asialoorosomucoid should block the activity of the ASF-DTA conjugate. This was the case (Fig. 3 *Lower*). As with ASF, asialoorosomucoid antagonized the ASF-DTA conjugate in a dose-dependent manner. Orosomucoid had no effect. When present from 10–1000 nM, asialoagalactoorosomucoid also had no effect. The slight apparent protection this derivative provided at 10 μM (this represents 10,000 molecules of competitor per molecule of ASF-DTA conjugate) could be accounted for if the asialoagalactoorosomucoid were contaminated with 0.25% of a partially galactosylated species. That figure is based on the amount of asialoagalactoorosomucoid required to give protection equivalent to that afforded by asialoorosomucoid (Fig. 3).

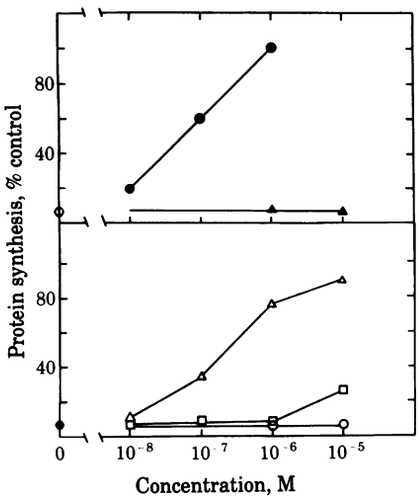


FIG. 3. Effects of sialic acid, galactose, and *N*-acetylglucosamine-terminated glycoproteins on the toxicity of the ASF-DTA conjugate. The glycoproteins were serially diluted in culture medium and a 1:100 vol of a 0.1 μM stock solution of ASF-DTA was added to give a final concentration of 1 nM. (*Upper*) ASF-DTA conjugate (1 nM) alone (♦), ASF-DTA + ASF (●), ASF-DTA + fetuin (▲). (*Lower*) In a separate experiment. ASF-DTA conjugate (1 nM) alone (♦), ASF-DTA + orosomucoid (○), ASF-DTA + asialoorosomucoid (△), ASF-DTA + asialoagalactoorosomucoid (□).

It has been shown that mannose-terminated glycoproteins are recognized by receptors present in reticuloendothelial cells and sinusoidal cells of the liver (for review, see ref. 46). Mannose-6-phosphate-terminated glycoproteins are recognized by fibroblast lysosomal and cell-surface receptors (46). Yeast mannan and phosphomannan are recognized by those respective receptors. We tested the ability of a high concentration (0.5 mg/ml) of these polysaccharides to block the toxicity of the ASF-DTA conjugate (1 nM). Neither had any effect on the action of the conjugate (data not shown).

Cell-Type Specificity. Functional ASGP receptors have been demonstrated *in situ* only in mammalian liver and in cell culture only in rat hepatocytes. No other tissue or established cell strain has been reported to possess functional ASGP receptors. Thus, only primary mammalian hepatocytes should be sensitive (in culture systems) to the ASF-DTA conjugate. We tested three established cell strains and primary cultures from three tissues, including the rat hepatocytes, for their sensitivity to the ASF-DTA conjugate (Table 1). The various cell types exhibited different sensitivities to diphtheria toxin. As expected, mouse 3T3 cells and primary rat cells from heart or liver were relatively insensitive to diphtheria toxin. The other cell lines were quite sensitive, with ED_{50} values below 100 pM. When challenged with 10 nM ASF-DTA conjugate, none of the cell types experienced *any* inhibition of protein synthesis, except for the primary rat hepatocytes. In this experiment, the ED_{50} for the hepatocytes was 50 pM.

DISCUSSION

We chose the ASGP receptor as a target for a cell-type-specific toxic conjugate because (*i*) no established or primary cell line other than primary mammalian hepatocytes (or intact liver) express a functional ASGP receptor and (*ii*) the mechanism of ligand–receptor internalization in this system has been well-characterized. This receptor, unique to a single cell type, has a known and relatively well-characterized function, although the physiological relevance of its action is not as yet understood (but see ref. 44). Although we cannot conclude from our data that no other cell type besides primary rat hepatocytes is sensitive to the ASF-DTA conjugate, the fact that five other cell types were unaffected by high concentrations of conjugate suggests a high degree of cell-type specificity. The insensitivity of those cells is particularly striking in view of the potency (on hepatocytes) of the ASF-DTA conjugate.

That carbohydrate specificity is the basis of the recognition of the ASF-DTA conjugate by the hepatocyte cell-surface receptors is firmly established by the glycoprotein competition studies. Thus, of the three forms of orosomucoid used, which differed only in their terminal carbohydrate residue(s), two (orosomucoid, asialoagalactoorosomucoid) did not block the activity of the ASF-DTA conjugate; in contrast, galactose-terminated asialoorosomucoid was an effective competitor, as would be expected from the carbohydrate specificity of the ASGP receptor.

Because the ASGP receptor is able to distinguish molecules on the basis of a single alteration in carbohydrate sequence, a

comparison of the toxicity of the ASF-DTA conjugate versus the F-DTA conjugate can distinguish ASGP receptor-mediated toxicity from toxicity due to other routes of internalization. This type of experiment has not been possible previously because the chemical basis for specificity has not been as structurally well-defined in any other receptor–ligand systems reported to date. About 300 times more F-DTA conjugate than ASF-DTA conjugate is required to achieve the same degree of inhibition of protein synthesis in hepatocytes (Fig. 2). The inability of fetuin or ASF to substantially block the action of the F-DTA conjugate suggests that the action of the F-DTA conjugate is not receptor-mediated. If we assume the non-ASGP-receptor-mediated uptake mechanism for ASF-DTA and F-DTA (which differ only in their terminal carbohydrate residues) are the same, then less than 1% of the toxicity of the ASF-DTA conjugate is due to non-ASGP-receptor-mediated routes of internalization. Although it is difficult to compare the effects of one conjugate with others prepared in different laboratories and assayed under various conditions, our results with the F-DTA conjugate suggest that caution should be exercised in concluding that the action of conjugates of low potency is receptor-mediated.

In recent studies, Hubbard and coworkers (22, 23, 25) have used electron microscopy to delineate the liver cell-type target, kinetics of internalization, and intracellular fate of ^{125}I-labeled and also ferritin- and horse radish peroxidase-conjugated ASGPs. They showed that ^{125}I-labeled ASF is bound rapidly and specifically by liver parenchymal cells (1–2 min after injection in rats) and is internalized (1–5) min. They concluded from their experiments with lactoperoxidase and ferritin conjugates of asialoorosomucoid that ASGPs are probably internalized in coated vesicles (25). Over half of the internalized ^{125}I-labeled protein becomes associated with lysosomes by 30 min (23). Of particular interest was their observation that about 40% of the internalized protein did not go to lysosomes but remained in the cytosol in undefined structures (23). If the ASF-DTA conjugate is internalized by the same pathway, it is possible that the active ASF-DTA conjugate is that material which escapes degradation in such a manner.

Because of the information already available on the mechanism by which ASGPs are internalized by the hepatic ASGP receptor, the ASF-DTA conjugate system should be valuable in delineating the variables in productively introducing DTA into cells. The unique occurrence of the ASGP receptor in hepatocytes should be of great value in optimizing site-directed killing of target tissues *in vivo* by toxic conjugates.

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