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Toxic Ligand Conjugates as Tools in the Study of Receptor-Ligand Interactions

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We have constructed hybrid proteins in which the toxic A chains of ricin or diptheria toxin have been linked to either asialofetuin, fetuin, or epidermal growth factor (EGF). Both ASF-RTA and ASF-DTA are potent toxins on cultured rat hepatocytes, cells that display the asialoglycoprotein receptor. Toxicity of these two compounds is restricted to hepatocytes and can be blocked by asialoglycoproteins but not the native glycoproteins or asialoagalactoglycoprotein derivatives, indicating that the toxicity of the conjugates is mediated by the hepatic asialoglycoprotein receptor. The EGF-RTA conjugate is an extremely potent toxin on cells that can bind the hormone, but is only poorly effective on cells that are unable to bind EGF. The EGF-DTA conjugate, in contrast, is unable to kill 3T3 cells and is at least two orders of magnitude less effective than EGF-RTA on A431 cells, a cell line with 1–2 $\times$ 10^6 EGF receptors per cell. However, when EGF-RTA and EGF-DTA were tested on primary liver hepatocyte cultures, which were susceptible to both ASF-RTA and ASF-DTA, both EGF conjugates were potent toxins. Sensitivity of the hepatocyte cultures to ricin toxicity increases slightly during a 52-hr culture period. In contrast, sensitivity to EGF-RTA and ASF-RTA decline dramatically during this period. Receptors for both ligands remain plentiful on the cell surface during this time.

Key words: epidermal growth factor, asialoglycoprotein receptor, ricin, diphtheria toxin, toxic conjugates, hybrid toxins, chimeric toxins

A number of plant and bacterial toxins kill sensitive cells by catalytically inhibiting protein synthesis. Although the mechanisms by which they inactivate the protein synthesis apparatus may differ, many of these toxins are similar in structure in that they are composed of a binding protein and a protein with enzyme activity. Ricin, a toxin isolated from the castor bean Ricinnus communis, is composed of two polypeptide chains. The B subunit (RTB) is a carbohydrate binding protein which is able to recognize and bind to galactose-terminal glycoproteins and glycolipids on the cell surface [1]. The A chain of the ricin molecule (RTA) inhibits protein synthesis

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by catalytically inactivating the larger subunit of ribosomes of susceptible species [2]. The two chains of the ricin molecule are joined by a single disulfide bond. Neither chain alone is a potent toxin. The B chain is able to bind to cells, but has no significant toxicity. Although the isolated A chain of ricin is able to catalytically inactivate ribosomes in vitro, it cannot efficiently bind to cell surfaces and traverse the membrane to gain access to the cytosol. Consequently, it is four to five orders of magnitude less toxic on cultured cells than intact ricin. The ricin molecule is a potent toxin only when the two subunits are joined together by the disulfide bond.

Diphtheria toxin, produced by Corynebacterium diphtheriae carrying the appropriate bacteriophage, is synthesized as a single polypeptide. It is, however, susceptible to limited proteolytic cleavage which yields two fragments, joined together by a disulfide bond. The B fragment (DTB) binds to the surface of target cells but cannot, by itself, serve as a toxin. The A fragment (DTA) inhibits the protein synthesis apparatus by catalytically transferring ADP-ribose from NAD to elongation factor 2 (EF2) [3]. The isolated DTA fragment, while able to inactivate EF2 by ADP-ribosylation in the cell-free system, is only a poor toxin on cells normally sensitive to diphtheria toxin. Potent toxicity is achieved only when the binding DTB fragment is joined by the disulfide to the enzymatically active DTA fragment.

Recently a number of laboratories have attempted to prepare conjugates or "chimeric" molecules between binding ligands and the catalytic subunits of ricin or diphtheria toxin. The objective has been to replace the B chain with a ligand of choice, and construct heteroconjugates with the binding specificity of the ligand and the catalytic toxicity of the toxin. Such heteroconjugates have a number of potential applications in biology and medicine, including the study of the process by which marcromolecules traverse the cell membrane, the receptor-directed elimination of target cells in mixed populations of cells in vivo or in culture, and the selection of variant cell lines defective in the binding and/or the internalization of specific ligands.

We have recently constructed hybrid proteins between the A chains of ricin or diphtheria toxin and two cell-surface binding ligands [4–6]. One of the experimental systems we have used takes advantage of the presence of receptors for desialyated (or asialo) glycoproteins present on hepatocytes. Ashwell, Morrell, and their co-workers demonstrated that desialylated serum glycoproteins are rapidly cleared from the circulation as a consequence of their binding to the hepatic asialoglycoprotein receptor and the subsequent receptor-mediated endocytosis and degradation of the asialoglycoprotein. The asialoglycoprotein receptor (ASGPR), a carbohydrate-binding protein which recognizes galactose-terminated glycoconjugates, has been found only on hepatocytes. This receptor, which requires at least two galactose-terminal residues per ligand for effective endocytosis, has been purified and extensively characterized (for review see [7]). Because of the wealth of information on the structure of the ASGP receptor and its cell biology this system should be an excellent model to study the action of toxic hybrid conjugates. We have constructed conjugates of asialofetuin (ASF) with both RTA and DTA, and characterized the specificity and toxicity of the ASF-RTA and ASF-DTA conjugates on cultured rat hepatocytes [5,6].

We have also prepared conjugates of RTA and DTA with epidermal growth factor (EGF) [4]. EGF is currently the most well characterized polypeptide mitogen, as evidenced by the large number of studies presented at this conference. EFG is a 53 amino acid polypeptide isolated from the submaxillary glands of male mice. There are no lysines in the protein; the only primary amino group is present at the amino

terminus of the molecule. Because the conjugation chemistry we have used requires the availability of a free amino group, the stoichiometry of the EGF-FTA and EGF-DTA conjugates is well defined. In this review we will summarize our studies with the hybrid conjugates of RTA and DTA coupled through disulfide linkages to either ASF or EGF.

MATERIALS AND METHODS

Isolation of Proteins

EGF was prepared from salivary glands of male mice [8]. Ricin was purified from castor beans by the method of Cawley et al [9]; RTA was subsequently isolated by elution with mercaptoethanol from Sepharose-bound ricin [4]. DTA was the gift of John Collier. Asialofetuin was prepared by desialylation of fetuin (Sigma) with *Clostridium perfringens* neuraminidase [4,5]. Orosomucoid, asialoorosomucoid, and asialoagalactoorosomucoid were gifts of M. Wickerhause (American Red Cross), J. Whitehead (Vector Labs) and J. Paulson (UCLA), respectively.

Cell Culture

Rat hepatocytes were isolated and cultured as described by Attie et al [10]. Cultures of bovine aortic endothelial cells [11] and rat heart cells [12] were prepared in the laboratories of Drs J. Berliner and I. Harary. Swiss 3T3 cells, A431 cells, and vero cells were maintained in Dulbecco's modified Eagle's medium and 5% fetal calf serum. Chinese hamster ovary cells (CHO-KII) were maintained in Ham's F12 medium and 5% fetal calf serum.

Preparation of Hybrid Protein Conjugates

Details of these reactions have been described previously [4–6]. In brief, the primary amino groups of fetuin, asialofetuin and EGF were derivatized with N-suiccinimidyl-3-(2-pyridyldithio) propionate (SPDP) from Sigma. The pyridyldithiopropionate (PDP) protein derivatives were separated from unreacted SPDP by chromatography on G-25 Sephadex. The derivatives were then carried through a thiol-disulfide interchange reaction with RTA or DTA, and the conjugates were isolated by gel-exclusion chromatography on appropriate Sephadex columns.

Protein Synthesis Assay

Cells were incubated for 24 hr in fresh medium containing various concentrations of toxin. After removing the toxin, cells were exposed for 2 hr to fresh medium containing either a ^{14}C amino acid mixture or ^{3}H-leucine. Monolayers were harvested and processed for determination of radioactivity. Data are presented as the percent incorporation relative to control cultures. The ED_{50} is the concentration of toxin required to inhibit protein synthesis 50% in a 2-hr pulse after 24 hr of exposure. Primary cell cultures were assayed in 17-mm 24-well cell culture dishes; cell lines were grown and assayed directly in scintillation vials [4–6].

Degradation of ^{125}I-ASF and ^{125}I-Fetuin

Approximately 1.5×10^6 or 6×10^5 3T3 cells, grown in 35-mm multiwell dishes, were exposed to 2 ml of medium containing either ^{125}I-fetuin or ^{125}I-ASF (0.2

μg/ml; 4.4 nM). The undegraded protein was precipitated from the medium by 2% phosphotungstic acid:10% trichloroacetic acid and the radioactivity in the supernatant was measured. [6,10].

Binding of ^{125}I-EGF to Hepatocytes and 3T3 Cells

Purified EGF was labeled with ^{125}I by the cloramine-T method. Specific activity was 80 μCi/μg. The procedure used to measure binding of ^{125}I-EGF to cultured cells at 4°C has been published [13].

RESULTS

Preparation of ASF-DTA, ASF-RTA, and Fetuin-DTA Conjugates

The primary amino groups of fetuin and asiolofetuin were derivatized with SPDP using a molar ratio of SPDP:protien of 2.5, to give an average of 1 mol of reagent/mol protein. After removing the unreacted reagent, reduced DTA was added to give an equimolar ratio of DTA:ASF-PDP and DTA:fetuin-PDP, and the thiol-disulfide interchange reaction was carried out. The ASF-DTA and fetuin-DTA conjugates were then separated from the unreacted proteins by chromatography on Sephadex G100 [5]. Analysis of the conjugates by SDS-acrylamide gel electrophoresis demonstrated that the major reaction products were 1:1 conjugates of ligand and DTA. Preparation of ASF-RTA was carried out in a similar fashion, with the exception that the ASF-PDP derivative contained an average of 4–5 mol of PDP/mol ASF, the consequence of using a 15-fold excess of SPDP in the initial conjugation reaction. Polyacrylamide gel analysis of the thiol-disulfide interchange reaction product showed the ASF-RTA conjugate produced under these conditions to be a 1:1 conjugate [6]. ASF-RTA, ASF-DTA, and fetuin-DTA were all dissociated to their respective subunits by reducing agents [5,6].

Characterization of Primary Rat Hepatocyte Cultures

Hepatocytes prepared in our laboratory by the method of Attie et al [10] were able to bind, internalize, and degrade ^{125}I-ASF but not ^{125}I-fetuin. In contrast, 3T3 cells, which do not have the ASGP receptor, were unable to degrade either protein (Table I). These hepatocyte cultures should, therefore, be susceptible to killing by ASF-RTA or ASF-DTA.

Toxicity of the ASF-RTA and ASF-DTA Conjugates on Cultured Hepatocytes

Ricin is a potent toxin on cultured hepatocytes, with an Ed_{50} between 10^{-12}–10^{-11}M (Fig. 1). In contrast, diphtheria toxin, known to be only slightly toxic to rodent cells [14,15], did not kill primary rat hepatocytes. As expected, the isolated A chains of ricin and diphtheria toxin were also unable to efficiently kill the cultured rat hepatocytes. In contrast, both ASF-RTA and ASF-DTA were potent toxins in these cells (Fig. 1). When ASF was mixed with equimolar amounts of RTA or DTA and applied to these cultures no significant toxicity occurred (data not shown). The ASF must be joined by a disulfide bond to the catalytic toxin A chain in order to efficiently direct target-cell killing. It should be noted that toxicities of the intact toxins and the hybrid proteins vary over about a ten-fold range, presumably due to differences in culture conditions and cell physiology.

TABLE I. Degradation of Asialofetuin and Fetuin by Cultured Rat Hepatocytes and 3T3 Cells

Cell	Degradation rate (fmol/min/10^6 cells)	
	^{125}I-asialofetuin	^{125}I-fetuin
Hepatocytes	1–2	< 0.1
3T3 cells	< 0.1	< 0.1

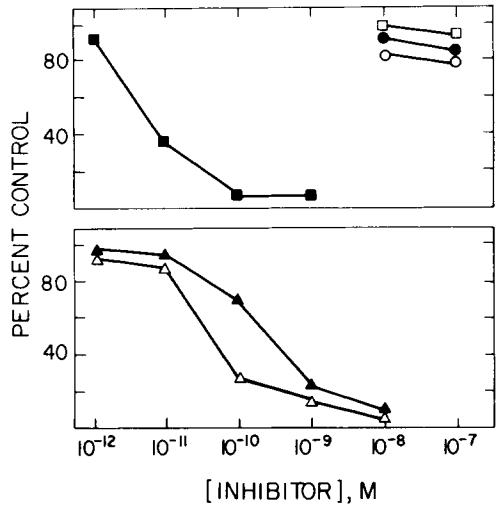

Fig. 1. Inhibition of protein synthesis in cultured rat hepatocytes by ASF-RTA and ASF-DTA: Data are expressed as percent of untreated controls. Ricin (■); RTA (□); Diphtheria toxin (●); DTA (○); ASF-RTA (△); ASF-DTA (▲). Reproduced from Simpson et al [6].

To demonstrate that killing is mediated by ASGP receptors we utilized competition with native and modified glycoproteins. Native glycoproteins such as fetuin and orosomucoid should not be able to block the toxicity of ASF-DTA or ASF-RTA. In contrast, asialofetuin and asialoorosomucoid (ASOM) should be effective competitors for binding of ASF-RTA and ASF-DTA to the ASGP receptor and should, therefore, protect cultured hepatocytes from the toxicity of ASF-RTA and ASF-DTA. These predictions were met completely (Table II). While fetuin and orosomucoid were unable to inhibit any toxicity of ASF-RTA or ASF-DTA at the highest concentration tested (10^{-5}M), the desialylated derivatives of these molecules were effective inhibitors. At concentrations of 10^{-6}–10^{-5}M ASF and ASOM could completely block the toxicity of ASF-RTA or ASF-DTA [5,6]. The terminal galactose of asialo serum glycoproteins can be removed enzymatically to produce N-acetyl-glucosaminyl-terminated asialo agalactoglycoprotein derivatives. If the toxicity of the ASF-A chain conjugates is mediated by the ASGP receptor the asialo agalacto glycoprotein should not be able to block toxicity. This was the case; asialo-agalacto orosomucoid (ASAGOM) was an ineffective inhibitor of ASF-DTA toxicity. At 10^{-5}M ASAGOM could block only 25% of the toxicity of ASF-DTA. At this concentration there are 10^4 molecules of competitor present for each molecule of ASF-DTA. This slight protection by ASAGOM could be completely accounted for by only a 0.25% contamination by ASOM, ie, if the galactosidase reaction was less than 99.75% complete.

TABLE II. Antagonism of Toxicity of ASF-RTA and ASF-DTA* by Native and Modified Glycoproteins

Glycoprotein	Concentration (M) required for 50% antagonism of toxicity of ASF-RTA	ASF-DTA
Fetuin	NI[a]	NI
Asialofetuin	3×10^{-7}	10^{-7}
Orosomucoid	NI	NI
Asialoorosomucoid	10^{-7}	5×10^{-7}

*ASF-RTA and ASF-DTA were used at 10^{-9} M.
[a]NI, no inhibition.

Hepatocytes are the only cell type known to express the ASGP receptor [7]. The ASF-RTA and ASF-DTA conjugates should, therefore, not be toxic to other types of cultured cells. ASF-DTA toxicity was tested on three primary cultures and three continuous cell lines (Table III). Only hepatocytes were susceptible to killing by ASF-DTA; the other cell lines and primary cultures were unaffected by concentrations of ASF-DTA 200 times greater than the ED_{50} for primary hepatocyte cultures.

Toxicity of Fetuin-DTA on Cultured Hepatocytes

This hepatocyte-specific receptor system offers a unique opportunity to examine the degree of specificity conferred by a receptor-ligand interaction in the study of toxic conjugates. Fetuin, which differs from asialofetuin only in its terminal carbohydrate residue, is not recognized by the ASGP receptor. A comparison of the toxicity of fetuin-A chain conjugates with ASF-A chain conjugates and free A chains should be quite informative in determining the receptor-specificity of the conjugates. When ASF-DTA and fetuin-DTA were titered in a single experiment, their ED_{50} values were 0.13 nM and 40 nM, respectively. Fetuin-DTA was thus 300 times less effective than ASF-DTA in inhibiting protein synthesis in hepatocytes. While it is possible that the low level of toxicity of the fetuin-DTA conjugate might be due to a trace of partially desialylated fetuin in the preparation (0.3% would be sufficient), the toxicity of the fetuin-DTA conjugate could not be blocked by ASF, suggesting that this low level of toxicity exhibited by fetuin-DTA was due to non-ASGP receptor-mediated toxicity.

Preparation of EGF-RTA and EGF-DTA Conjugates

The only free amino group on the EGF molecule is at the N-terminus; there are no lysine residues in the molecule [8]. The reaction with SPDP should, therefore, produce a derivative (EGF-PDP) with only a single site available for conjugation to RTA or DTA. EGF was derivatized with a five-fold molar excess of SPDP. The EGF-PDP derivative was isolated by chromatography on Sephadex G-25. The EGF-RTA and EGF-DTA conjugates were constructed by carrying out a thiol-disulfide exchange in which 1–2 mol of EGF-PDP were used per mole of A chain. EGF-RTA and EGF-DTA were separated from unreacted A chain and EGF-PDP by chromatography on Sephadex G-75. Comparison with molecular weight standards suggested that both conjugates had a 1:1 stoichiometry between A chain and EGF. After reduction both EGF-RTA and EGF-DTA were cleaved to their component polypeptides [4].

Toxicity of EGF-RTA 3T3 Cells

EGF-RTA is within an order of magnitude as potent a toxin as ricin on 3T3 cells (Fig. 2). When RTA is joined by a disulfide bond to either EGF or RTB it is four orders of magnitude more effective in killing 3T3 cells than when added to cells by itself. Reduction of plating efficiency and protein synthesis by toxins were proportional (data not shown). Equimolar mixtures of EGF and RTA are ineffective as a toxin; the two proteins must be joined together by a disulfide bond in order to kill 3T3 cells. Antiserum to the ricin B chain could effectively inhibit the toxicity of ricin, but had no effect on the toxicity of EGF-RTA. In contrast, antiserum to the ricin A chain was able to block the toxicity of both ricin and EGF-RTA. The toxicity of EGF-RTA should be blocked by unconjugated EGF if the conjugate is acting through the EGF receptor. When protein synthesis in control cultures was inhibited 95% by EGF-RTA, EGF blocked toxicity of EGF-RTA in a dose-dependent manner [4]. EGF-RTA should not be able to kill cells that are unable to bind EGF. We have previously isolated two 3T3 variant cell lines, 3T3-NR6 [16] and 3T3-TNR-2 [17] that are unable to bind detectable levels of EGF. 3T3-TNR-2 cells were compared with wild-type 3T3 cells for sensitivity to ricin and EGF-RTA. The two cell lines differed by a factor

TABLE III. Inhibition of Protein Synthesis in Various Cell Types by ASF-DTA*

Cell type	ED_{50},pM
Vero	> 10,000 (NI)[a]
3T3	> 10,000 (NI)
CHO	> 10,000 (NI)
Primary rat hepatocytes	50
Primary rat heart cells	> 10,000 (NI)
Primary bovine aortic epithelium cells	> 10,000 (NI)

*Cells were exposed to a series of concentrations of ASF-DTA conjugate ranging from 1–10,000 pM.
[a]NI, no inhibition.

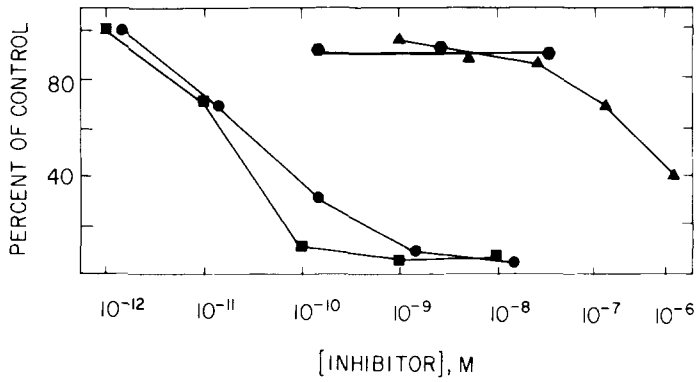

Fig. 2. Inhibition of protein synthesis in 3T3 cells by EGF-RTA, ricin, and RTA: Ricin (■); EGF-RTA (●); RTA (▲); equimolar mixtures of EGF and RTA (●). Reproduced from Cawley et al [4].

of at least 30-fold in sensitivity to EGF-RTA (Table IV). The toxicity of the EGF-RTA conjugate may be an even more sensitive assay for the presence of EGF receptors than the binding of labeled EGF.

Toxicity of EGF-DTA on 3T3 Cells

We next wanted to test the toxicity of diphtheria toxin and EFG-DTA on 3T3 cells, and compare these results with the toxicity of EGF-RTA. Diphtheria toxin was, as expected, unable to kill 3T3 cells (Fig. 3). (Diphtheria toxin is known to be ineffective on murine cells [14,15].) In contrast to the EGF-RTA conjugate, EGF-DTA was unable to kill 3T3 cells, even at concentrations as great as 3×10^{-8}M (Fig.3). The lack of toxicity of EGF-DTA on 3T3 cells could possibly be due to inactivation of the enzymic activity of the DTA moiety, or be the result of an alteration in the ability of the EGF moiety to recognize the EGF receptor. However, the EGF-DTA conjugate was able to carry out the ADP-ribosylation of murine EF-2 nearly as well as DTA. When EGF-RTA, EGF-DTA, and EGF were used as competitors for ^{125}I-EGF in a receptor-binding assay to 3T3 cells, all three competitors had an equivalent capacity to block ^{125}I-EGF binding. Moreover, the nontoxic EGF-DTA conjugate could block the toxicity of EGF-RTA on 3T3 cells [4] and could even act as a mitogen for 3T3 cells (data not shown). Despite an absence of toxicity, both the enzymic moiety and the binding moiety of the EGF-DTA conjugate are functional in the conjugate.

TABLE IV. Toxicity of Ricin and EGF-RTA on 3T3 and 3T3-TNR-2 Cells

		ED_{50} (M)	
Cell	EGF receptors per cell	Ricin	EGF-RTA
3T3	66,000	3×10^{-12}	1×10^{-10}
3T3-TNR-2	Not detectable	5×10^{-12}	$> 3 \times 10^{-8}$

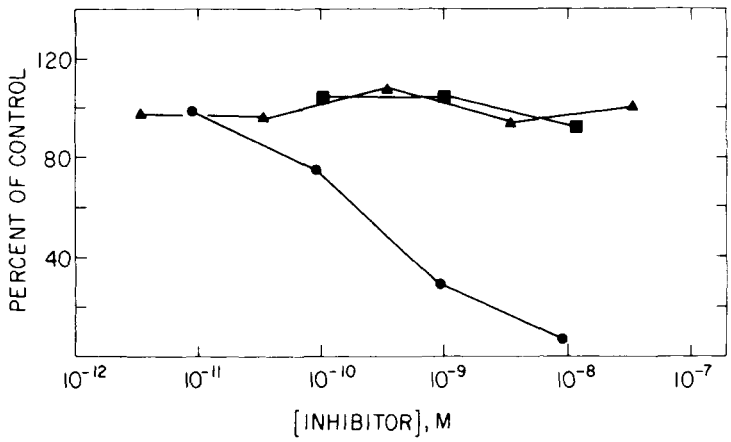

Fig. 3. Inhibitioin of protein synthesis in 3T3 cells by EGF-RTA, EGF-DTA, and diphtheria toxin: EGF-RTA (●); EGF-DTA (▲); diphtheria toxin (■). Reproduced from Cawley et al [4].

Toxicity of EGF-RTA and EGF-DTA on A431 Cells

The human epidermoid carcinoma cell line A431 has 1–2 × 10^6 EGF receptors per cell, 10–20 times the number of EGF receptors present on 3T3 cells [18]. These cells might, therefore, be more susceptible to toxicity by EGF-DTA. While toxicity of EGF-DTA on A431 cells could be demonstrated, EGF-RTA was over 100-fold more effective than EGF-DTA (Table V). The distinction in the toxicities of EGF-RTA and EGF-DTA observed on 3T3 cells is also seen on A431 cells.

Toxicity of EGF-RTA and EGF-DTA on Cultured Rat Hepatocytes

The difference in the toxicity of the DTA and RTA conjugates of EGF on 3T3 or A431 cells stands in striking contrast to the toxicity of the DTA and RTA conjugates of ASF on cultured hepatocytes. In the former case the RTA conjugate was much more effective than the corresponding DTA conjugate (Fig. 3, Table V). In the latter case both conjugates were about equally toxic (Fig. 1). Hepatocytes are reported to possess substantial numbers of EGF receptors [19]. We decided to test the two EGF conjugates, EGF-DTA and EGF-RTA, to determine whether the cell type may determine susceptibility to the conjugates.

Binding experiments demonstrated that our cultured hepatocytes had about three times the number of EGF receptors present on 3T3 cells (Fig. 4). When EGF-RTA and EGF-DTA were tested on cultured rat hepatocytes the results were in direct

TABLE V. Toxicity of EGF-RTA and EGF-DTA on A431 Cells

	ED_{50} (M)	
Cell type	EGF-RTA	EGF-DTA
3T3	5×10^{-11}	$> 3 \times 10^{-8}$
A431	7×10^{-12}	5×10^{-10}

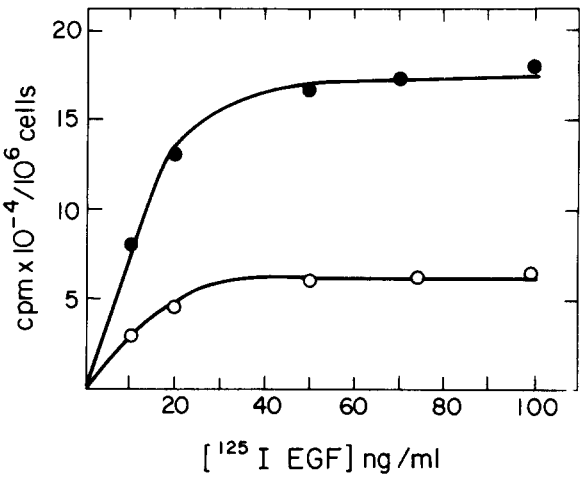

Fig. 4. Binding of ^{125}I-EGF to 3T3 cells and to cultured rat hepatocytes: Data are expressed as cpm ^{125}I-EGF bound per 10^6 cells. Hepatocytes (●); 3T3 cells (○).

contrast to what we observed on 3T3 or A431 cells. Both EGF conjugates were equally toxic on hepatocytes (Fig. 5). For purposes of comparison the data for ricin, ASF-RTA and ASF-DTA toxicity are reproduced in Figure 5. The toxicity of the two EGF conjugates is greater than that observed for the ASF conjugates and is, within the error of the assay, comparable to the toxicity of ricin.

Toxicity of EGF-RTA and ASF-RTA as a Function Time in Culture of Hepatocytes

Preliminary studies indicated that the sensitivity of primary hepatocyte cultures to toxic conjugates decreased with the age of the cultures. To examine this question more extensively we tested the toxicity of ricin, RTA, ASF-RTA, and EGF-RTA on a common set of hepatocyte cultures at three different times after plating. Cells were exposed to the toxins at 4, 28, and 52 hr after plating, and protein synthesis was assayed after 24 hr of exposure to toxin.

As expected, RTA was only slightly toxic at all times (Fig. 6A). The toxicity of ricin *increased* somewhat during the 52-hr culture period prior to toxin addition. The toxicity of EGF-RTA decreased as the cultures aged: over the 52-hr culture period prior to toxin addition the ED_{50} dropped from 2×10^{-11}M to 5×10^{-10}M for EGF-RTA (Fig. 6B). The toxicity of ASF-RTA decreased to the greatest extent, dropping from 4×10^{-10}M at 4 hr to 2×10^{-8}M, a 50-fold increase, at 24 hr. By 52 hr in culture, hepatocytes were essentially resistant to ASF-RTA, with an ED_{50} greater than 10^{-8}M (Fig. 6C).

One possible explanation for the reduced sensitivity of aging hepatocyte cultures to EGF-RTA and ASF-RTA is a loss of receptors for the ligands. Binding of ^{125}I-EGF to hepatocytes in culture for 4 and 28 hr was identical. After 52 hr in culture the binding of ^{125}I-EGF declined by about 35%. The ability to bind ^{125}I-ASF *increased* over two-fold in the 28-hr cultures, then decreased to about one-half the original value in 52-hr cultures (data not shown). The decrease in sensitivity of the hepatocyte cultures to EGF-RTA and ASF-RTA thus occurs at a much more rapid rate than the loss of receptors for the binding ligands.

DISCUSSION

The use of toxic conjugates in biology and medicine has only recently received a great deal of attention. The initial studies utilized intact toxins (diphtheria toxin, ricin) covalently linked to lectins, antibodies or Fab fragments. Only in the past five years have the isolated catalytic subunits of the toxins been used in conjunction with targeting ligands. While some notable successes have been achieved in constructing toxic conjugates, many of the chimeric molecules have been relatively poor toxins, despite retention of activity of both the binding moiety and the enzymic toxin. To date no predictive rules have emerged for the potential success or failure of a toxic conjugate. Our lack of success in developing general strategies for the construction and utilization of toxic conjugates reflects our lack of knowledge concerning the mechansims by which the ligands traverse the cell membrane, the manner in which the toxic subunit traverses the membrane, and the pathway by which the enzymatic subunit of the toxin gains access to the cytosolic compartment once it has entered the cells. Because so few toxin molecules are necessary to kill cells if they are presented

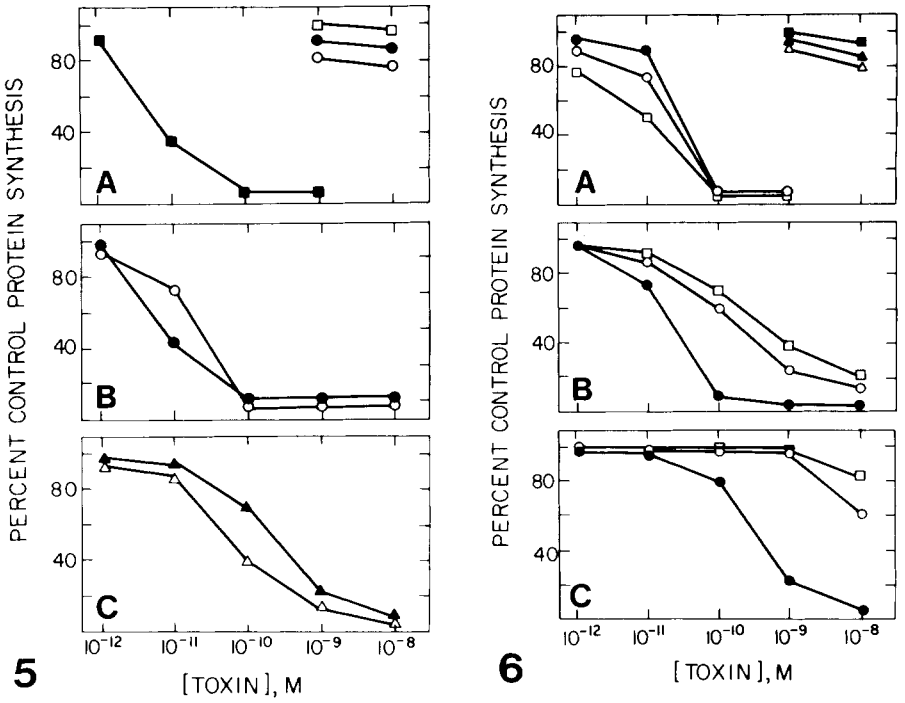

Fig. 5. Inhibition of protein synthesis in cultured rat hepatocytes by toxic conjugates, toxins, and A chains: Panel A: Ricin (■); RTA (□); DTA (○); diphtheria toxin (●). Panel B: EGF-RTA (○); EGF-DTA (●). Panel C: ASF-RTA (△); ASF-DTA (▲).

Fig. 6. Inhibition of protein synthesis by ricin, RTA, ASF-RTA, and EGF-RTA in hepatocytes cultures of increasing age: Toxins were added to hepatocytes previously in culture for 4, 28, or 52 hr. After a 24-hr exposure to toxin, protein synthesis was assayed. Incorporation into controls was 49,000 ± 6,000 cpm (4 hr); 55,000 ± 8,000 cpm (28 hr); and 40,000 ± 12,000 cpm (52 hr). In each case data are the average ± SD from 12 wells. Panel A: Ricin added to 4-hr (●), 28-hr (○), or 52-hr (□) cultures. RTA added to 4-hr (▲), 28-hr (△), or 52-hr (■) cultures. Panel B: EGF-RTA added to 4-hr (●), 28-hr (○), or 52-hr (□) cultures. Panel C: ASF-RTA added to 4-hr (●), 28-hr (○), and 52-hr (■) cultures. Reproduced from Simpson et al [6].

in the appropriate manner, it is difficult to study, either with biochemical or ultrastructural techniques, the nature of the "productive" internalization of the toxic A chains.

It would be useful, in studying the mechanisms of toxin-hybrids, if the systems under study would possess as many as possible of the following characteristics: (1) the ligand should be readily available, (2) the receptor should be well characterized, (3) the receptor should be restricted to one or a few cell types, (4) the mechanism by which the ligand is internalized should be well understood and finally (5) if possible, a probe for the receptor other than ligand binding should be available. The two systems we have chosen for study probably come closer to satisfying these criteria than most other receptor systems available. (1) Fetuin can be purchased, and asialofetuin can be prepared in gram quantities. EGF is among the easiest of the peptide growth factors to prepare. The unique chemistry of EGF (with a single primary amino

group) and its extraordinary stability confer additional advantages on this system. (2) The ASGP receptor can be purified in milligram quantities [7]. More is known about the biology of the EGF receptor than perhaps any receptor studied. (3) The ASGP receptor has been found only on hepatocytes. While the EGF receptor is widely distributed, variant cell lines have been described that are unable to bind to EGF [16,17] and cells with heroic levels of EGF receptors are available [18]. (4) The pathways of internalization of the ASGP ligand receptor complex and the EGF-receptor complex are probably the best characterized representatives of Kaplan's class I and class II receptors [20]. (5) Finally, conventional and monoclonal antibodies to both the ASGP and EGF receptors are now available as reagents to identify and isolate these receptors independent of their ligand-binding activity.

Both ASF and EGF enter the cell by a pathway that involves clustering of receptors, invagination into coated pits, and vesicle-mediated endocytosis of both the receptor and the ligand. We presume that the hybrid toxins constructed with these two ligands are, for the most part, taken into the cell "piggy-back" in endocytic vesicles, along with the appropriate receptor. If this is, indeed, the method by which *productive* internalization of the A chains occurs in these toxic conjugates, then we are confronted with the problem of how the A chains escape the vesicular compartment and gain access to the cytosol, where their substrates are found. Both ASF and EGF are transported in a vesicular form to the lysosomes, where they are degraded. If the toxic A chains enter the cell via the EGF or ASGP receptor then they must escape lysosomal degradation in order to be effective as toxins. We have found that chloroquine is able to increase the potency of the EGF-RTA conjugate six-fold [4] and reduce the degradation of the A chain (unpublished data), suggesting that protection from lysosomal degradation will enhance the opportunity of the RTA in the EGF-RTA conjugate to gain access to the cytosolic compartment. Hubbard et al [21] have reported that a significant portion of internalized ASF does not go to lysosomes. Perhaps that portion of the conjugate which is not directed to lysosomes is active in inhibition of protein synthesis.

It is, however, possible that the small number of A chain molecules responsible for inhibition of protein synthesis and cell death gain access to the cytosol by a pathway other than the receptor-mediated endocytosis of the directing ligand. There may be a distinct pathway by which the A chains, once brought in close proximity to the cell surface, can traverse the membrane; a pathway independent of the receptor-mediated endocytosis of the binding ligand. Such a pathway for internalization, independent of the binding ligand once the A chain has come in appropriate contact with the membrane, may also be active in the productive internalization of A chains of intact toxin. If this event occurs infrequently relative to receptor-mediated endocytosis via the binding ligand it would be difficult to detect and characterize. Should this, indeed, be the mechanism by which the major activity of the intact toxins or the heteroconjugates occurs, then the toxic conjugates would be useful in isolating receptorless variants for the binding ligands, but would probably not be as useful in selecting variants deficient in internalization of the binding ligand. Should productive internalization of the A chain require an internalization pathway independent of the ligand internalization pathway, the former event might very well occur in the absence of the latter. However, it should be noted that EGF-RTA and ASF-RTA, two of the most toxic heteroconjugates described to date, are constructed with binding ligands that are subject to very rapid receptor-mediated endocytosis once they have bound to the cell surface.

Neville and his colleagues have suggested that toxic conjugates made with ricin will require the presence of an intact B chain, to interact with an intracellular receptor for galactose, if the conjugate is to have potent toxicity [22,23]. They have derived their hypothesis primarily from studies with chemically modified monophosphate pentamannose derivatives of ricin. They predicted that asialoglycoprotein conjugates would require the presence of the ricin B chain in order to be highly toxic [23]. Our data, however, demonstrate that ASF-RTA is a potent toxin. Moreover, EGF-RTA is among the most toxic of the hybrid conjugates prepared. Clearly, productive introduction of the A chain of ricin can occur in these cases in the absence of B chain.

Probably the most intriguing aspect of the results we have described is the distinction in toxicity of EGF-DTA on 3T3 cells and A431 cells on the one hand and hepatocytes on the other. Uchida et al [24,25] described RTA and DTA conjugates of the *Wisteria floribunda* lectin and found that the RTA conjugate was a much more potent toxin than the DTA conjugate. These data are similar to our results for EGF-RTA and EGF-DTA on 3T3 or A431 cells. Both Uchida et al [25] and we [4] postulated that the hydrophobic sequences present in RTA [26] might permit RTA to become associated with the plasma membrane once the binding ligand had brought it to the appropriate location and proximity. In contrast, DTA does not contain any long stretches of hydrophobic sequences [27]. Consequently the internalization of DTA was postulated by both laboratories to be more dependent on the binding ligand. However, our studies with the ASF conjugates of RTA and DTA demonstrated that these two A chains, bound to a common ligand, have essentially equivalent toxicities for hepatocytes. Gilliland et al [28] found that the RTA and DTA conjugates of a monoclonal antibody to an antigen present on colorectal carcinoma cells had equivalent toxicities. In these systems, therefore, the hydrophobicity differences between RTA and DTA have no effect on the toxicity of the conjugates. Even more strikingly, EGF-DTA—not toxic to 3T3 cells and far less toxic than EGF-RTA on A431 cells—was as potent a toxin as EGF-RTA on hepatocytes. Clearly, the distinction in toxicity for EGF-DTA vs EGF-RTA on 3T3 cells and A431 cells must reside in the cell and not the polypeptides, since both conjugates are effective toxins on one cell type (hepatocytes) but display disparate toxicities on these cell types. The difference cannot be due to differences in numbers of EGF receptors, since hepatocytes—equally sensitive to EGF-DTA and EGF-RTA—have EGF receptors intermediate in numbers between 3T3 and A431 cells. Hepatocytes may recognize and process EGF-receptor complexes in a fashion different from 3T3 cells and A431 cells, which permits the DTA to escape into the cytosol. Alternatively, a secondary, non-EGF receptor-mediated pathway for productive DTA internalization may be available on hepatocytes but not on the other two cell types. These results emphasize the possibility of distinct cell-type restricted pathways for the internalization or processing of common ligands and/or A chains. These considerations have not previously received sufficient attention in discussions of toxic conjugates.

The decrease in sensitivity to EGF-RTA and ASF-RTA observed in hepatocytes with time in culture may be useful in unraveling the basis for toxicity of hybrid conjugates. Sensitivity to ricin increased over the same period, demonstrating both that the protein synthesis machinery remains sensitive to RTA action and that productive internalization of A chain can occur in these cells as they age in culture. Receptor numbers for either EGF or ASF did not decline in a level sufficient to account for the decreased sensitivity. Some aspect distal to receptor binding, such as receptor clustering, ligand internalization, and/or processing may change with age in these cells.

Methods for studying internalization and processing of these two ligands have been extensively characterized. Elucidation of time-dependent alterations in these processes might help to clarify the requisite steps in receptor-ligand cellular interactions necessary for productive presentation of A chain in heteroconjugate toxins.

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