Preparation and Characterization of Interleukin-2-Gelonin Conjugates Made Using Different Cross-Linking Reagents[1]

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Conjugates of IL-2 with the ribosome-inactivating protein gelonin were prepared using heterobifunctional reagents to link the proteins *via* disulfide, acid-labile, and noncleavable linkers. In each case, one protein was modified using 2-iminothiolane. The sulfhydryl groups so introduced were then reacted either with 2-nitro-5-dithiobenzoate groups or with iodoacetamido groups which had been introduced into the second protein. In the case of the acid-labile linkage, a reagent which forms a labile bond upon reaction with amino groups, 4-(iodoacetamido)-1-cyclohexene-1,2-dicarboxylic acid anhydride (its synthesis is described in this paper) was used to modify the toxin. The conjugates were separated from nonconjugated proteins by gel filtration on Sephadex G100 (SF). Each was analyzed with respect to its ribosome-inactivating activity, its ability to bind to the IL-2 receptor, and its *in vitro* cytotoxicity. The ribosome-inactivating activity of gelonin was unaffected by modification with 2-iminothiolane and was retained in conjugates prepared using this ragent. Modification of the toxin with 4-(iodoacetamido)-1-cyclohexene-1,2-dicarboxylic acid anhydride to form the acid-labile link drastically reduced the activity of the toxin. However, the activity of the toxin was recovered following acid treatment to release the native protein. Conjugates containing each type of linkage exhibited both specific binding and selective cytotoxicity toward cells expressing the IL-2 receptor. The most potent of these toxins, that containing the disulfide linkage, exhibited a cytotoxicity which was 2 orders of magnitude greater than that of unconjugated gelonin.

INTRODUCTION

A specific cytotoxic agent for selective killing of activated T cells would be of major therapeutic value in the treatment of T cell leukemia (Krönke *et al.*, 1985, 1986) and autoimmune diseases such as rheumatoid arthritis (Paliard *et al.*, 1991) and for the prophylaxis and treatment of graft-versus-host disease in tissue transplantation (Derocq *et al.*, 1987; Vitetta and Uhr, 1984; Kupiec-Weglinski *et al.*, 1988). Natural toxins from plants and bacteria, when conjugated to specific monoclonal antibodies, provide a powerful tool for selective elimination of particular populations of cells by allowing target-specific delivery of cytotoxins (Blättler *et al.*, 1989; Vitetta *et al.*, 1987). The ribosome-inactivating proteins (Olsnes and Pihl, 1982; Stirpe and Barbieri, 1986; Lambert *et al.*, 1988) seem to be ideal toxic agents for this purpose. Most effort has been directed toward using the plant protein ricin (Olsnes and Pihl, 1982; Frankel *et al.*, 1986; Vitetta *et al.*, 1987). This protein consists of two nonidentical subunits (A and B chains) that are joined by a disulfide bond (Olsnes and Pihl, 1982). The B chain has the property of binding to cell surface carbohydrates and promotes the uptake of the A chain into cells. Entry of the A chain into the cytoplasm results in the death of the cell by catalytic inactivation of its ribosomes (Endo *et al.*, 1987).

The single-chain ribosome-inactivating proteins have properties and characteristics similar to those of the ricin A chain alone (Stirpe and Barbieri, 1986; Endo *et al.*, 1988). They are highly effective at inactivating ribosomes in cell-free systems but are relatively nontoxic to intact cells because of lack of a B chain activity. These proteins are good candidates for the preparation of cytotoxic conjugates and have several advantages over ricin A chain: they are extremely stable proteins, resistant to proteolysis, and safe to purify without the extreme safety precautions necessary for work with ricin (Stirpe and Barbieri, 1986). They have been widely used in recent years for the preparation of immunotoxins (Thorpe *et al.*, 1981; Lambert *et al.*, 1985; Lambert *et al.*, 1988; Vitetta *et al.*, 1987).

Ligands other than antibodies can be used in order to deliver toxins to defined cell populations. Indeed, cytotoxic conjugates prepared using ligands such as thyrotropin-releasing factor (Bacha *et al.*, 1983), insulin (Miskimins and Shimizu, 1979), chorionic gonadotropin (Oeltman, 1985; Oeltman and Heath, 1979), leutenizing hormone (Singh *et al.*, 1989), epidermal growth factor (Cawley *et al.*, 1980; Shimizu *et al.*, 1980; Simpson *et al.*, 1982), transferrin (Raso and Basala, 1984), and α_2-macroglobulin (Martin and Houston, 1983) exhibit specific cytotoxicity toward cells expressing the relevant receptors. The availability of recombinant lymphokines has prompted us to consider the usefulness of one of these ligands as an agent for targeting ribosome-inactivating proteins to T cells. We describe here the preparation of conjugates consisting of the T-cell growth factor, interleukin-2 (Smith, 1980), and the single chain ribosome-inactivating protein, gelonin (Stirpe *et al.*, 1980). Conjugates were prepared using disulfide, acid-labile, and noncleavable linkages. Each conjugate was analyzed for its ribosome-inactivating activity, its ability to bind to the IL-2[2]-receptor, and its *in vitro* cytotoxicity.

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EXPERIMENTAL PROCEDURES

Materials. With the exception of the materials listed below, all reagents were obtained either from the sources described in the appropriate references or from Sigma Chemical Co., St. Louis, MO.

Recombinant IL-2 (human) was generously supplied by Biogen, Inc., Cambridge, MA. 2-Iminothiolane was purchased from Pierce Chemical Co., Rockford, IL. Gelatin (EIA grade) was from Bio-Rad Laboratories, Richmond, CA. L-[U-^{14}C]Cysteine·HCl (32.5 mCi/mol) was from Amersham Corp., Arlington Heights, IL, and was diluted to a specific radioactivity of 0.5 mCi/mmol with nonradiolabeled cysteine·HCl before use. Labeled Bolton–Hunter reagent (*N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]-iodophenyl)propionate; 2000 Ci/mmol) was also purchased from Amersham. [*Methyl*-^{3}H]thymidine (4 Ci/mmol) was obtained from ICN Biomedicals, Inc., Costa Mesa, CA. A rabbit reticulocyte lysate system for cell-free protein synthesis, which included L-[3,4,5-^{3}H]leucine (specific radioactivity 146.5 Ci/mmol), was obtained from New England Nuclear, Boston, MA, as was Biofluor scintillation cocktail. Betafluor scintillation cocktail was from National Diagnostics, Somerville, NJ. *N*-Succinimidyl iodoacetate (which is available from Sigma Chemical Co.) was synthesized by Mr. B. Kuenzi in our laboratories.

Seeds from *Gelonium multiflorum* were from United Chemical and Allied Products, 10 Clive Row, Calcutta-1, India, and were obtained through the Meer Corp., North Bergen, NJ.

C57 BL/6 female mice and Lewis rats were purchased from Jackson Laboratories, Bar Harbor, ME. The animals were maintained in accordance with the guidelines of the Committee of Animal Care of the Dana-Farber Cancer Institute and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. [NIH] 78-23, revised 1978). Rat and murine splenocytes were prepared as described by Kleiman *et al.* (1984).

The CTLL-2 cell line (Baker *et al.*, 1979) was a generous gift of Dr. Kendall Smith, Darthmouth Medical School, Darmouth, NH.

Synthesis of 4-(Iodoacetamido)-1-cyclohexene-1,2-dicarboxylic Acid.[3] 1,4-Cyclohexadiene-1,2-dicarboxylic acid di-*tert*-butyl ester was prepared from butadiene and acetylenedicarboxylic acid di-*tert*-butyl ester as described by Weber *et al.* (1980) and was then subjected to a standard hydroboration reaction with diborane in tetrahydrofuran to give 4-hydroxy-1-cyclohexene-1,2-dicarboxylic acid di-*tert*-butyl ester: ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 18H), 1.66–2.62 (m, 7H), 3.95 (m, 1H); IR (neat) ν_{max} 3430, 1710, 1645.

4-Amino-1-cyclohexene-1,2-dicarboxylic acid di-*tert*-butyl ester could be prepared from this alcohol in three steps: The alcohol (7.14 g, 23.9 mmol) and *p*-toluenesulfonyl chloride (6.49 g, 33.5 mmol) were dissolved in dry pyridine (43 mL), and the solution was stirred at ambient temperature overnight. Water was then added (0.66 mL), and stirring was continued for another 0.5 h. The solution was then concentrated in the cold under reduced pressure and the residue taken up into ether and washed successively with cold 1 M H_2SO_4, 1 M $NaHCO_3$, and water. After drying and removal of the ether, the crude tosylate was dried under high vacuum for 4 h and then dissolved in dry acetonitrile (90 mL). Sodium azide (1.94 g, 200 mmol) and 15-crown-5 (0.95 g, 4.3 mmol) were added, and the solution was refluxed overnight under N_2(g). The cooled solution was then filtered and concentrated to an oil which was redissolved in dry ether (100 mL) and subjected to a second filtration. Removal of the ether afforded a yellowish oil which was dissolved in dry methanol (75 mL) for the reduction of the azido group. 1,3-Propanedithiol (6.49 g, 60 mmol) and triethylamine (6.07 g, 60 mmol, distilled from 1-naphthyl isocyanate) were added, and the mixture was stirred at room temperature under N_2(g) for 40 h. The reaction mixture was then filtered and the filtrate concentrated on an aspirator with gentle heating, yielding an orange oil which was purified by flash chromatography on silica gel. The column (200 mL) was first washed with $CHCl_3$ (500 mL) and $CHCl_3$–MeOH (95:5 v/v, 300 mL) and then eluted with $CHCl_3$–MeOH (80:20 v/v). The pure amine was recovered as a colorless oil in 56% yield (3.96 g, 13.3 mmol): ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 18H), 1.6–2.6 (m, 8H), 2.97 (m, 1H); IR (neat) ν_{max} 3360, 1710, 1650.

The amine (297 mg, 1 mmol), iodoacetic acid (186 mg, 1 mmol), and *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (250 mg, 1 mmol) were dissolved in dry dichloromethane (5 mL), and the solution was stirred under N_2(g) at room temperature overnight. The reaction mixture was then poured into cold 0.1 M HCl, and the product was extracted with ethyl acetate. The combined extracts were washed with water, dried, and concentrated yielding pure 4-(iodoacetamido)-1-cyclohexene-1,2-dicarboxylic acid di-*tert*-butyl ester as an oil, which solidified to a brittle foam upon drying under high vacuum (452 mg, 0.97 mmol): ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 18H), 1.6–2.7 (m, 7H), 3.66 (s, 2H), 6.26 (d, J = 8 Hz, 1H). The di-*tert*-butyl ester (0.412 mg, 0.88 mmol) was converted with trifluoroacetic acid (4 mL) quantitatively into the diacid 4-(iodoacetamido)-1-cyclohexene-1,2-dicarboxylic acid: ^{1}H NMR (Me_2SO-d_6) δ 1.2–2.8 (m, 7H), 3.6 (s, 2H), 8.3 (d, J = 8 Hz, 1H), 9.7 (broad s, 2H).

Preparation of Conjugates. IL-2 was supplied by Biogen, Inc., as a solution (0.91 mg/mL) in 50 mM sodium acetate buffer, pH 3.5. Prior to conjugation, this material was dialyzed exhaustively at 4 °C against distilled water and was then buffered by the addition of triethanolamine base, HCl, and disodium EDTA from stock solutions so that the final concentration of buffer was 20 mM at pH 8.0 containing 1 mM EDTA. Gelonin was purified from the seeds of *Gelonium multiflorum* as described by Stirpe *et al.* (1980) and was stored frozen at −70 °C at a concentration of 5–6 mg/mL in 10 mM potassium phosphate buffer, pH 7.2, containing NaCl (145 mM). Stock solutions of 2-iminothiolane were prepared as described previously (Lambert *et al.*, 1978).

Conjugates of IL-2 with gelonin (Figure 1) were prepared using the synthetic routes described below. With the exception of the reactions used to introduce the functional groups into the proteins, the procedure for conjugation of IL-2 with gelonin, and for the purification of the conjugate, was the same in each case and is described in detail only for conjugate A.

Conjugate A (Disulfide Link). A solution of IL-2 (0.8 mg/mL) in 20 mM triethanolamine·HCl, pH 8.0, containing EDTA (1 mM) was degassed and then treated with 2-iminothiolane (2 mM) at 0 °C for 45 min under nitrogen. The excess reagent was removed by gel filtration

[2] Abbreviations used: IL-2, interleukin-2; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; CTLL, cytolytic T lymphocyte line; DMEM, Dulbecco's modified Eagles medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate.

[3] Blättler, W. A., and Lambert, J. M. (1988) U.S. Patent No. 4,764,368. Acid-cleavable compound.

at 4 °C on Sephadex G25 (fine) equilibrated with 50 mM triethanolamine·HCl, pH 8.0, containing EDTA (1 mM). The conditions described here result in the addition of 0.7–0.9 sulfhydryl groups per IL-2 molecule. Gelonin (5–6 mg/mL) was diluted to 4 mg/mL with distilled water, 0.5 M triethanolamine·HCl, pH 8.0, and 0.1 M disodium EDTA, pH 8.0, so that the final concentrations of triethanolamine and EDTA were 60 and 1 mM, respectively. The solution was degassed and then treated with 2-iminothiolane (1 mM) at 0 °C for 45 min under nitrogen. The excess reagent was removed by gel filtration at 4 °C on Sephadex G25 (fine) equilibrated with 5 mM bis-Tris·acetate, pH 5.8, containing NaCl (50 mM) and EDTA (1 mM). Under these conditions, 0.6–0.8 sulfhydryl groups were introduced per gelonin molecule. The modified gelonin was treated with 5,5′-dithiobis(2-nitrobenzoic acid) (10 mM) at 4 °C for 30 min in order to form the mixed disulfide of the modified gelonin and 2-nitro-5-thiobenzoate. This material was purified by gel filtration at 4 °C on Sephadex G25 (fine) equilibrated with 50 mM triethanolamine·HCl, pH 8.0, containing EDTA (1 mM). Conjugate A was prepared by incubating the mixed disulfide (0.3 mg/mL final concentration) with a slight molar excess of the modified IL-2 at 4 °C for 16 h under nitrogen, after which the reaction mixture was treated with iodoacetamide (1 mM) at 4 °C for 1 h in order to block any remaining free sulfhydryl groups. The conjugate was purified by gel filtration at 4 °C on Sephadex G100 (superfine) equilibrated with 20 mM bicine·NaOH, pH 8.5, containing betaine (250 mM). Fractions containing pure conjugate were pooled and dialyzed against bicine·NaOH, pH 8.5, containing NaCl (150 mM). The conjugate was then concentrated to 0.12 mg/mL using a Millipore CX10 microconcentrator, frozen in small aliquots (250 μL) using liquid nitrogen, and stored at −70 °C.

Conjugate B (Acid-Labile Link). Sulfhydryl groups were introduced into IL-2 using 2-iminothiolane as described above. Gelonin was modified using 4-(iodoacetamido)-1-cyclohexene-1,2-dicarboxylic acid anhydride. The reagent was prepared immediately before use by treating 4-(iodoacetamido)-1-cyclohexene-1,2-dicarboxylic acid (0.125 M) in dimethyl sulfoxide with dicyclohexylcarbodiimide (0.15 M) at 25 °C for 1 h, and following filtration the solution was used without further purification. Gelonin (5–6 mg/mL), diluted to 4 mg/mL with 100 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM), was treated with the anhydride (312.5 μM, assuming complete reaction of the dicarboxylic acid) at 25 °C for 15 min. Excess reagent was removed by gel filtration at 4 °C on Sephadex G25 (fine) equilibrated with 50 mM triethanolamine·HCl, pH 8.0, containing EDTA (1 mM). Under these conditions, approximately 0.8 iodoacetamido groups were introduced per gelonin molecule.

Conjugate C (Noncleavable Link). Iodoacetamido groups were introduced into IL-2 using *N*-succinimidyl iodoacetate. A solution of IL-2 (0.8 mg/mL) in 50 mM triethanolamine·HCl, pH 8.0, containing EDTA (1 mM) was treated with *N*-succinimidyl iodoacetate (208 μM), added from a 40 mM solution in dioxane, at 30 °C for 15 min. The reaction was terminated by gel filtration at 4 °C on Sephadex G25 (fine) equilibrated with 50 mM triethanolamine·HCl, pH 8.0, containing EDTA (1 mM). About 0.9 iodoacetamido groups were introduced per IL-2 molecule. Sulfhydryl groups were introduced into gelonin using 2-iminothiolane as described above.

Conjugate D (Noncleavable Link: Toxin Inactive). Sulfhydryl groups were introduced into IL-2 using 2-iminothiolane as described above. Gelonin (5–6 mg/mL) was diluted to 4 mg/mL with 100 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM) and was then treated with *N*-succinimidyl iodoacetate (200 μM) at 30 °C for 15 min. Excess reagent was removed by gel filtration at 4 °C on Sephadex G25 (fine) equilibrated with 50 mM triethanolamine·HCl, pH 8.0, containing EDTA (1 mM). The conditions described here result in the introduction of about 0.7 iodoacetamido groups per gelonin molecule.

Measurement of Extent of Protein Modification. Sulfhydryl groups introduced into the proteins were quantified spectrophotometrically by the method of Ellman (1959). The incorporation of iodoacetamido groups into the proteins was measured by incubating samples of the modified proteins (0.2–1.2 mg/mL) with excess [^{14}C]-cysteine (1.7 mM; 0.5 mCi/mmol) in 50 mM triethanolamine·HCl buffer, pH 8.0, containing EDTA (1 mM) for 1 h at 25 °C. The labeled proteins were separated from [^{14}C]cysteine by gel filtration at 4 °C on columns (4 mL) of Sephadex G25 (fine) equilibrated with 50 mM triethanolamine·HCl, pH 8.0, containing EDTA (1 mM). Fractions (0.2 mL) were counted in 4 mL of Biofluor scintillation cocktail using a Packard Tri-Carb Model 2000 CA scintillation counter (efficiency for ^{14}C was 90%).

Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of 0.1% (w/v) SDS was carried out in acrylamide gel slabs (145 mm × 90 mm × 0.75 mm) prepared according to Laemmli (1970). Samples of protein in 25 μL of buffer, pH 8.5, were prepared for electrophoresis by mixing with urea (28 mg) and 3 μL of a solution of SDS (20% w/v) containing iodoacetamide (100 mg/mL) and incubating at room temperature for 1 h. The urea was necessary to ensure complete denaturation of proteins in the absence of heating, and iodoacetamide was added to react with any free sulfhydryl groups (Lambert *et al.*, 1978). Proteins were stained with Coomassie Brilliant Blue R250.

Measurement of Protein Concentration. Concentrations of purified proteins were determined from their A_{280nm}, using $E^{1\%}_{1cm}$ values of 6.7 for gelonin (Stirpe *et al.*, 1980) and 7.0 for IL-2 [the latter value was measured in a solution of IL-2 whose concentration was determined by two different methods using bovine serum albumin as a standard (Lowry *et al.*, 1951; Bradford, 1976)]. The protein concentrations of the purified conjugates were also estimated from their A_{280nm}, using an $E^{1\%}_{1cm}$ value of 6.8.

Ribosome-Inactivating Activity of the Conjugates. The inhibitory activity of gelonin toward protein synthesis was measured in a rabbit reticulocyte lysate system. The assay was based on that of Pelham and Jackson (1976), using materials provided by New England Nuclear supplemented with additional reagents as described in detail previously (Lambert *et al.*, 1985; Lambert and Blättler, 1988).

Binding Studies. IL-2 was labeled using the method of Bolton and Hunter (1973). IL-2 (5 μg) was reacted with 250 pmol of *N*-succinimidyl 3-(4-hydroxy-5[^{125}I]iodophenyl)propionate in 10 μL of 0.1 M sodium borate buffer, pH 8.5, at 0 °C for 15 min. In order to prevent the subsequent labeling of carrier proteins, the reaction solution was then treated at 0 °C for 15 min with 200 μL of 0.5 M glycine in 0.1 M sodium borate, pH 8.5. Labeled IL-2 was purified by gel filtration at 4 °C on Sephadex G25 (fine) equilibrated with 50 mM sodium phosphate buffer, pH 7.5, containing 0.25% (w/v) gelatin. The pure sample had a specific radioactivity of 18.8 μCi/μg.

CTLL-2 cells were maintained in exponential growth in DMEM supplemented with 40% (v/v) conditioned medium (prepared using rat splenocytes as described by Gillis

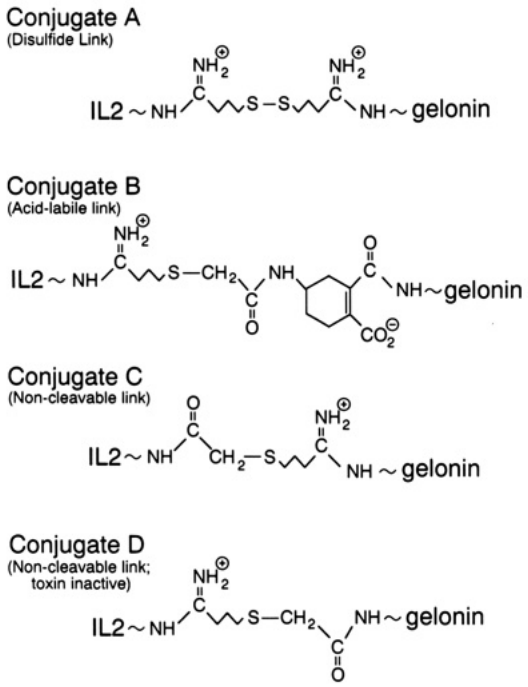

Figure 1. IL-2–gelonin conjugates using different types of linkers.

et al. (1978)), fetal calf serum (10% (v/v)), penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL).

Binding assays were carried out in RPMI 1640 medium containing 25 mM HEPES buffer, pH 7.2, and 10 mg/mL of bovine serum albumin (assay buffer). CTLL-2 cells (70 × 10^6 cells) were washed three times with 50 mL of assay buffer at room temperature, incubated with 50 mL of the buffer for 1 h at 37 °C, and then washed a further three times at room temperature (Robb *et al.*, 1981). Dilutions of IL-2 or of conjugate were prepared in final volumes of 100 μL of assay buffer containing 5 × 10^5 CTLL-2 cells and 7.0 fmol of ^{125}I-labeled IL-2. The level of nonspecific binding was determined by including mixtures which contained an excess of unlabeled IL-2 (500 pmol). The mixtures were incubated for 1 h at 4 °C in 0.7 mL Eppendorf tubes after which 0.4 mL of assay buffer was added, and the cells were pelleted at 13 000 rpm for 2 min at 4 °C. The cells were washed twice with 0.5 mL of ice-cold assay buffer, and bound label was then measured using an LKB Model 1272 γ counter.

Proliferation Assays. Proliferation assays were carried out in 96-well flat-bottom microtiter plates in RPMI 1640 medium supplemented with fetal calf serum (10% (v/v)), penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL). Dilutions of IL-2 or of the conjugates were prepared in triplicate in 200 μL of the above medium containing 0.5 × 10^6 murine splenocytes and either concanavalin A (1 μg/mL) or lipopolysaccharide (1 μg/mL). Mixtures containing no added IL-2 or conjugate were also prepared. The plates were incubated for 48 h at 37 °C, and each well was pulsed with 1 μCi of [*methyl*-^{3}H]thymidine for the final 4 h of the incubation. The cells were harvested on glass fiber filters using a PHD harvester (Cambridge Biotechnology, Inc., Cambridge, MA), and the incorporated label was counted in 4 mL of Betafluor scintillation cocktail.

RESULTS

Preparation and Purification of Conjugates. Four conjugates of IL-2 with gelonin (Figure 1) were prepared using the synthetic routes described in the Experimental Procedures. In each case, the two proteins were modified to introduce complementary reactive groups and were then cross-linked in a reaction which gave only the heterodimer in high yield.

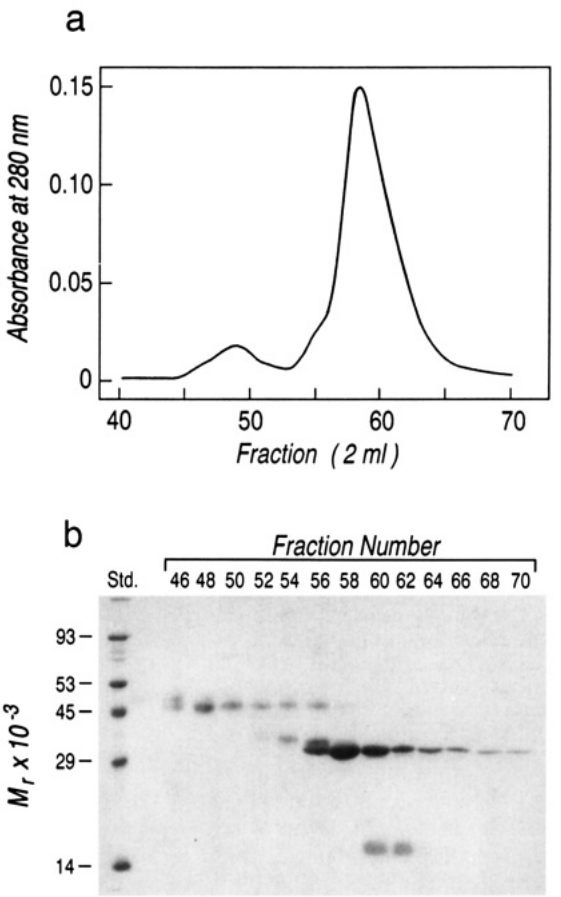

Figure 2. Gel filtration of an IL-2-gelonin conjugation reaction mixture and analysis of the elution profile by SDS–polyacrylamide gel electrophoresis. IL-2 and gelonin were conjugated as described in the Experimental Procedures for the preparation of conjugate D. Panel a: elution profile of the conjugation mixture (2.2 mg of protein in 2 mL) submitted to gel filtration through a column (99 cm × 1.6 cm) of Sephadex G100 (superfine) equilibrated with 20 mM bicine·NaOH buffer, pH 8.5, containing 250 mM betaine. Panel b: 12.5% (w/v) SDS–polyacrylamide gel of samples taken from fractions of the elution profile shown in panel a. The gel was run under nonreducing conditions as described in the Experimental Procedures, and the calibration of M_r was from the mobility of proteins of known subunit M_r (Std): phosphorylase b (93 000), glutamate dehydrogenase (53 000), ovalbumin (45 000), carbonic anhydrase (29 000), and lysozyme (14 300).

In order to minimize the formation of high molecular weight polymeric conjugates, the proteins were modified to introduce on average only 0.6 to 0.9 reactive groups per molecule. The conditions required for the introduction of mercaptobutyrimidoyl groups into gelonin using 2-iminothiolane have already been described (Lambert *et al.*, 1985). For each of the other modification reactions, preliminary experiments were carried out to determine the conditions required to introduce the appropriate number of reactive groups into either IL-2 or gelonin.

Examination of the conjugation reaction mixtures by SDS–polyacrylamide gel electrophoresis (not shown) demonstrated that a major product of each reaction was a heteroconjugate (M_r ~46 000) consisting of one IL-2 molecule (M_r 15 000; Liang *et al.*, 1985) linked to one gelonin molecule (M_r 30 500; Thorpe *et al.*, 1981; Lambert *et al.*, 1988) as inferred from the mobility of the stained protein bands. Bands corresponding in apparent molec-

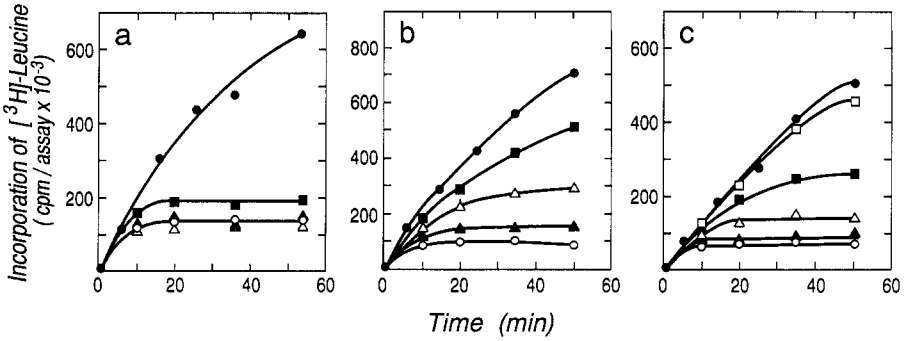

Figure 3. Effect of the modification of gelonin on its inhibitory activity toward protein synthesis in a cell-free system from rabbit reticulocyte lysates. Protein synthesis was measured by the incorporation of [^{3}H]leucine into protein precipitable with trichloroacetic acid as described the Experimental Procedures. The incorporation of radioactivity in the controls within 50 min was in the range of 500 000–700 000 cpm per assay (volume 27 μL) and was counted with an efficiency of 20–25% (Packard Tri-Carb Model 2000 CA scintillation counter). Samples of gelonin modified to introduce iodoacetamido groups and the corresponding control samples of nonmodified gelonin were treated with 2-mercaptoethanol (1 mM) for 1 h at 25 °C before assay. All samples were maintained under reducing conditions prior to the assay by the inclusion of dithioerythritol (2 mM) in the sample buffer. Panel a: inhibition of protein synthesis by gelonin modified using 2-iminothiolane: (●) control assay; (○) assay that contained 20 pg of gelonin; (▲, △, ■) assays that contained 20 pg of gelonin modified with 2-iminothiolane to introduce 0.5 sulfhydryl groups per molecule of gelonin (▲), 1.14 groups per gelonin (△), or 2.93 groups per gelonin (■). Panel b: inhibition of protein synthesis by gelonin modified using 4-(iodoacetamido)-1-cyclohexene-1,2-dicarboxylic acid anhydride: (●) control assay; (○) assay that contained 20 pg gelonin; (▲, △, ■) assays that contained 20 pg of gelonin modified with 0.49 iodoacetamido groups per molecule of gelonin (▲), 0.66 groups per gelonin (△), or 1.90 groups per gelonin (■). Panel c: inhibition of protein synthesis by gelonin modified using *N*-succinimidyl iodoacetate: (●) control assay; (○) assay that contained 20 pg of gelonin; (▲, △, ■, □) assays that contained 20 pg of gelonin modified with *N*-hydroxysuccinimidyl iodoacetate to introduce 0.56 iodoacetamido groups per molecule of gelonin (▲), 0.9 groups per gelonin (△), 1.38 groups per gelonin (■), or 2.69 groups per gelonin (□).

ular weight to small amounts of IL-2–gelonin conjugates with IL-2 to gelonin stoichiometries of 1:2 and 2:1 could also be seen in the gels. Other products of the conjugation reaction included small quantities of cross-linked gelonin and cross-linked IL-2 which likely result from the formation of protein–protein disulfide bonds following the modification of either IL-2 or gelonin using 2-iminothiolane. In addition, IL-2 contains a nonbonded sulfhydryl group (Liang *et al.*, 1985) which, although not sufficiently reactive to be of use in the preparation of conjugates (J.M. Lambert, unpublished results), may participate in the formation of disulfide cross-linked IL-2.

The most effective way to obtain samples of pure IL-2–gelonin was found to be a single step of gel filtration on Sephadex G100 (superfine). Under conditions of low ionic strength, there was a significant interaction between IL-2 and gelonin which resulted in some contamination of the purified conjugate with unconjugated gelonin. This was minimized by the inclusion of 250 mM betaine in the buffer used for gel filtration. Figure 2a shows the elution profile obtained when the reaction mixture obtained in the preparation of conjugate D was purified in this manner. Similar profiles were obtained for each of the other conjugation reaction mixtures. Two incompletely separated peaks of protein were obtained. Analysis of the peaks by SDS–polyacrylamide gel electrophoresis (Figure 2b) showed that the first peak (fractions 45–53) consisted primarily of the IL-2–gelonin conjugate (M_r ~46 000), although bands that may corresponds to small small amounts of IL-2 dimer and trimer contaminated the trailing and leading edges of the peak. The second peak consisted of unconjugated gelonin (peak fraction 58) and IL-2 (peak fraction, 61).

The yields of conjugate, based on densitometric analysis of samples analyzed by SDS–polyacrylamide gel electrophoresis, were in the order of 15–20% with respect to IL-2. Purified material for biological testing was obtained by pooling only those fractions which contained no contaminating proteins. This material, which represented less than 60% of the total conjugate formed, was at least 95% pure as judged by SDS–polyacrylamide gel electrophoresis.

Effect of Modification of Gelonin on Its Ribosome-Inactivating Activity. The ability of gelonin to inactivate ribosomes was measured by dilution of samples into an assay of cell-free protein synthesis using the rabbit reticulocyte system. Figure 3 shows the effects of chemical modification on the ribosome-inactivating activity of gelonin. Under the conditions used, 20 pg of native gelonin completely inhibited protein synthesis within 10–20 min. Modification of gelonin using 2-iminothiolane had little effect on the catalytic activity of the toxin (Figure 3a); even the introduction of 2.9 groups caused only a minimal reduction in the ribosome-inactivating activity of the protein. In contrast, modification with either 4-(iodoacetamido)-1-cyclohexene-1,2-dicarboxylic acid anhydride (Figure 3b) or with *N*-succinimidyl iodoacetate (Figure 3c) resulted in a significant decrease in the enzymatic activity of the protein. Comparison of these results with assays carried out with further dilutions of native gelonin indicated that the modification of an *average* of 1 residue per gelonin molecule using these reagents resulted in a 50–70% reduction in the activity of the toxin (this result indicated to us that it was possible that the *modified* molecules of gelonin were completely inactivated).

Ribosome-Inactivating Activity of the Conjugates. The ribosome-inactivating activity of the purified conjugates was also measured using the cell-free assay system. Both conjugates which had been prepared using gelonin modified with 2-iminothiolane (conjugates A and C) exhibited significant ribosome-inactivating activity, although the activity was reduced relative to that of native gelonin by about 60–80% as estimated by comparison with assays carried out with serial dilutions of native gelonin (Lambert and Blättler, 1988). However, preincubation of the conjugate formed with a disulfide linkage (conjugate A) with dithioerythritol released fully active gelonin that was indistinguishable from native gelonin in its ability to inhibit protein synthesis in these assays (Figure 4a). As expected, the activity of conjugate C was unaffected by preincubation with dithioerythritol (result not shown).

The ability of gelonin to inhibit protein synthesis was drastically reduced in purified preparations of both conjugate D and conjugate B. Whereas protein synthesis

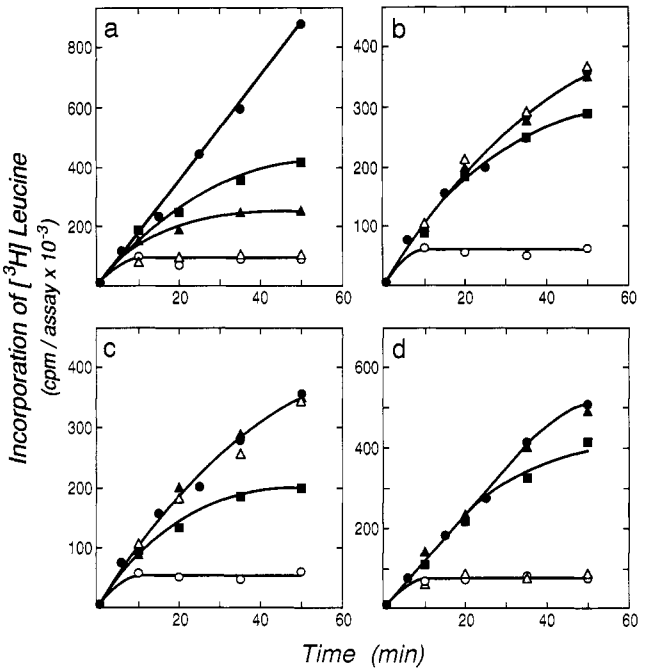

Figure 4. Inhibition of protein synthesis in a cell-free system from rabbit reticulocyte lysates by gelonin or by conjugates of IL-2 with gelonin. Protein synthesis was measured by the incorporation of [^{3}H]leucine into protein precipitable with trichloroacetic acid as described in the Experimental Procedures. The incorporation of radioactivity in the controls within 50 min was in the range of 350 000–900 000 cpm per assay (27 μL) and was counted with an efficiency of 20–25% (Packard Tri-Carb Model 2000 CA scintillation counter). Panel a: (●) control assay; (○) assay that contained 20 pg of gelonin; (▲, △) assays that contained an amount of conjugate A (disulfide link) that contained 20 pg of gelonin without (▲) and with (△) pretreatment of the conjugate with dithioerythritol (20 mM) for 30 min at 30 °C; (■) assay that contained an amount of conjugate C (noncleavable link) equivalent to 20 pg of gelonin. Panel b: (●) control assay; (○) assay that contained 20 pg of gelonin; (▲, △, ■) assays that contained an amount of conjugate D (noncleavable link) equivalent to 20 pg (▲), 200 pg (△), or 2 ng (■) of gelonin. Panel c: (●) control assay; (○) assay that contained 20 pg of gelonin; (▲, △, ■) assays that contained an amount of conjugate B (acid-labile link) equivalent to 20 pg (▲), 200 pg (△), or 2 ng (■) of gelonin. Panel d: (●) control assay; (○) assay that contained 20 pg of gelonin; (▲) assay that contained an amount of conjugate B equivalent to 20 pg of gelonin; (△, ■) assays that contained an amount of conjugate B equivalent to 20 pg of gelonin after incubation of the conjugate (23 μg/mL) for 48 h at 37 °C, either in 100 mM MES·HCl buffer, pH 5.5, containing 0.1 mg/mL of bovine serum albumin (△) or in 100 mM sodium phosphate buffer, pH 7.2, containing 0.1 mg/mL of bovine serum albumin (■).

was completely abolished in 10–15 min by 20 pg of native gelonin, an amount of conjugate D equivalent to 2 ng of gelonin failed to completely inhibit protein synthesis, even after 50 min (Figure 4b). Similarly, amounts of conjugate B (acid-labile link) corresponding to 2 ng of gelonin were required for complete inhibition of protein synthesis which was achieved only after about 40 min, while 200 pg of conjugated gelonin failed to cause any inhibition of protein synthesis in these assays (Figure 4c). In the case of the acid-labile conjugate B, however, the release of gelonin by incubation of the conjugate in MES·HCl buffer, pH 5.5, at 37 °C for 48 h completely restored the ability of the gelonin to inhibit protein synthesis (Figure 4d). A control treatment of the conjugate at pH 7.2 and 37 °C for 48 h yielded less than 20% of the ribosome-inactivating activity of native gelonin.

Binding of the Conjugates to CTLL-2 Cells. The ability of the conjugates to bind to the IL-2 receptor was measured by competitive displacement of ^{125}I-labeled IL-2 from CTLL-2 cells. Figure 5 shows a series of binding curves comparing the binding of the four conjugates with that of nonconjugated IL-2. Under the conditions used (see Experimental Procedures), 50% of the label specifically bound by the cells was displaced by 400 pM IL-2. The displacement curves for the conjugates indicate that the attachment of gelonin to IL-2 has reduced the binding affinity by about 1 order of magnitude, concentrations of conjugate of between 3 and 7 nM being required to displace 50% of the label bound to the IL-2 receptor.

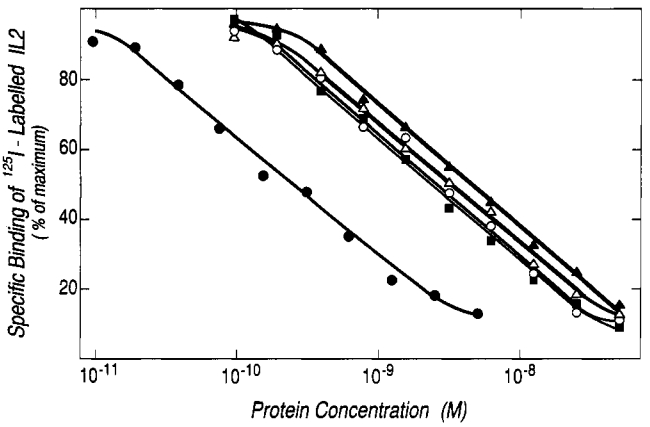

Figure 5. Competitive inhibition of the binding of ^{125}-I-labeled IL-2 to CTLL-2 cells by IL-2 or by conjugates of IL-2 with gelonin. Binding experiments were carried out at 4 °C as described in the Experimental Procedures. Under the conditions of the assay, 50% of the labelled IL-2 (3.5 fmol; 2200 cpm) bound to the cells in the absence of competing ligand. Nonspecific binding represented 7% of the total binding capacity of the cells. The curves show the inhibition of the binding to label to CTLL-2 cells by IL-2 (●), conjugate A (○), conjugate B (▲), conjugate C (△), and conjugate D (■).

Inhibition of T-Cell and B-Cell Responses. The inhibition of lymphocyte proliferative responses, as measured by the inhibition of incorporation of [^{3}H]thymidine relative to controls, was used as a measure of cytotoxicity. Concanavalin A was used as a T-cell mitogen. Conjugate A, in which IL-2 was linked to gelonin through a disulfide bond, clearly inhibited the proliferative response of murine splenocytes to concanavalin A (Figure 6a). The IC_{50} of the conjugate, ~500 pM, is 2 orders of magnitude lower than the IC_{50} for native gelonin (50 nM). An admixture of IL-2 and gelonin showed only the same cytotoxicity as gelonin alone. The acid-labile and noncleavable conjugates were less effective than conjugate A. The IC_{50} of the noncleavable conjugate C was only 10-fold lower than the IC_{50} of native glonin, and the effect of the acid-labile conjugate B on the T-cell response was indistinguishable from that of the unconjugated toxin.

Concanavalin A induces expression of IL-2 receptors and production of IL-2 by mouse lymphocytes (Robb *et al.*, 1981). Growth of the activated lymphocytes is maintained by stimulation of the cells by autocrine and paracrine IL-2. Under these conditions, it is possible that the IL-2–gelonin conjugates might inhibit the proliferative response of lymphocytes to concanavalin A by direct competition for binding to the IL-2 receptor rather than by the inhibition of protein synthesis. This possibility was examined using conjugate D in which the ability of gelonin to inhibit protein synthesis was reduced by several orders of magnitude. This conjugate showed no effect on the proliferative response of the lymphocytes at any concentration tested (Figure 6a), indicating that the effects of the other conjugates were dependent on the presence

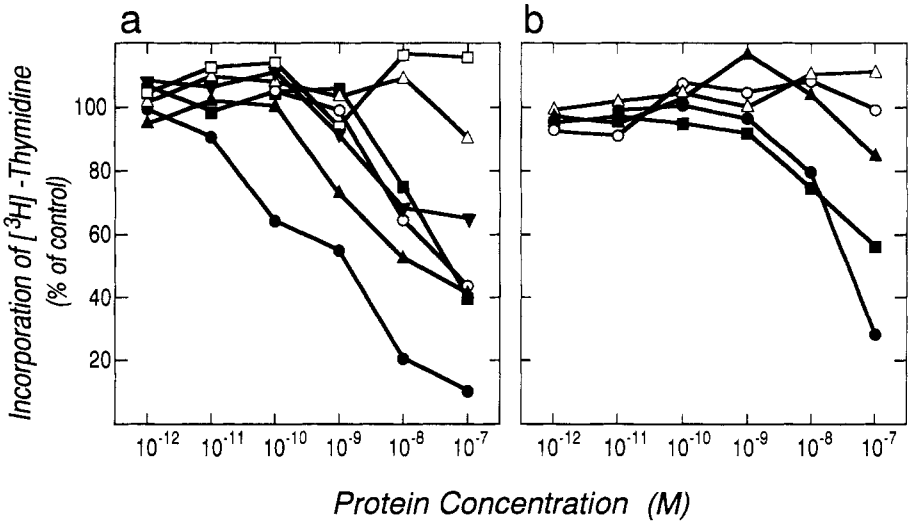

Figure 6. Cytotoxicity of conjugates of IL-2 with gelonin. The inhibition of proliferative responses in lymphocytes as measured by the inhibition of the incorporation of [^{3}H]thymidine relative to controls was used as a measure of cytotoxicity as described in Methods. Panel a: inhibition of T-cell response; murine splenocytes were stimulated with concanavilin A and incubated for 48 h in the presence of conjugate A (●), conjugate B (○), conjugate C (▲), conjugate D (△), gelonin (■), IL-2 (□), or an admixture of IL-2 and gelonin (▼). Panel b: inhibition of B cell response; murine splenocytes stimulated with lipopolysaccharide and incubated for 48 h in the presence of conjugate A (●), conjugate B (○), conjugate C (▲), conjugate D (△), or gelonin (■).

of the ative toxin and were not simply due to competition of binding at the level of the IL-2 receptor.

In order to confirm the selectivity of the conjugates for cells expressing the IL-2 receptor, their effect on the proliferative response of lymphocytes to the B-cell mitogen lipopolysaccharide was examined. Figure 6b shows that the proliferative response of murine splenocytes to lipopolysaccharide was not affected by the acid-labile conjugate or by the two non-cleavable conjugates. The conjugate of IL-2 linked to gelonin through a disulfide bond did exhibit some toxicity in this assay system, but only over the range of concentrations at which native gelonin itself was toxic.

DISCUSSION

Studies with immunotoxins have established that the nature of the linkage used in the preparation of such conjugates, the amount of binding to the cell surface, and the extent of internalization of the conjugates are important factors influencing their cytotoxicity toward target cells (Lambert *et al.*, 1985; Goldmacher *et al.*, 1989). Single-chain ribosome-inactivating proteins, such as gelonin, do not bind to the cell surface and rely on fluid-phase pinocytosis as their only mode for internalization (Goldmacher *et al.*, 1986). When gelonin is conjugated to monoclonal antibodies that recognize antigens which are present in high numbers on the cell surface, and which are internalized efficiently, the cytotoxicity of the conjugates may be 2–4 orders of magnitude greater than that of the unconjugated toxin (Thorpe *et al.*, 1981; Lambert *et al.*, 1985; Lambert *et al.*, 1988; Goldmacher *et al.*, 1989).

In this paper, we describe conjugates between IL-2 and gelonin prepared with disulfide, acid-labile, and non-cleavable linkers. The most potent of these conjugates, that containing a disulfide bond, exhibited a cytotoxicity which was 2 orders of magnitude greater than that seen with unconjugated gelonin, as judged by the effect of the conjugate on the proliferative response of murine splenocytes to concanavalin A. *In vitro* assays showed that cleavage of the disulfide bond restored the full catalytic activity of the toxin which may account, in part, for the observation that the conjugate containing the disulfide bond (conjugate A) was more effective in inhibiting T cell function than that prepared using a noncleavable link (conjugate C). The cleavable disulfide linkage may also allow the toxin to escape more readily into the cytoplasm from the membrane-bound IL-2/IL-2 receptor complex.

As previously reported (Lambert *et al.*, 1985), gelonin was not affected by modification with 2-iminotholane in its ability to inhibit protein synthesis. In contrast, modification of the toxin with either *N*-succinimidyl iodoacetate or with 4-(iodoacetamido)-1-cyclohexenyl-1,2-dicarboxylic acid anhydride resulted in a drastic reduction in its ability to inactivate ribosomes. The reason for the striking difference in the effects of modifying gelonin using these cross-linking reagents is not clear. One could speculate that these reagents react preferentially with different amino groups on gelonin, one of which is important for catalytic activity. Another factor is the preservation of the positive charge of amino groups upon reaction with 2-iminothiolane. If this were important for maintaining full catalytic activity, then modification with *N*-succinimidyl iodoacetate or with 4-(iodoacetamido)-1-cyclohexenyl-1,2-dicarboxylic acid anhydride, each of which react with amino groups to form neutral amides, might be expected to inactivate the toxin. In this respect, it is interesting to note that modification of gelonin with other cross-linking reagents which form uncharged amide bonds, for example, *N*-hydroxysuccinimide esters such as *N*-succinimidyl 3-(2-pyridyldithio)propionate, also reduce its ability to inactivate ribosomes (Thorpe *et al.*, 1981; Blättler *et al.*, 1985; Senter *et al.*, 1985).

The conjugate of gelonin coupled to IL-2 through a noncleavable thioether linkage (conjugate D) showed no cytotoxicity, as expected, since the catalytic activity of the toxin was almost completely abolished. When gelonin was linked to IL-2 through the acid-labile linkage (conjugate B) the ribosome-inactivating activity of the gelonin in this conjugate was also almost completely abolished. However, the catalytic activity of the toxin from conjugate B was fully restored following release of the native toxin by incubation under mildly acidic conditions (pH 5.5), as expected from previous work with analogous reagents (Kirby and Lancaster, 1970; Blättler *et al.*, 1985). In spite of this, the cytotoxicity observed for conjugate B was no more than that seen with unconjugated gelonin, although

it is generally assumed that many ligands bound to cell-surface receptors are internalized into acidified vesicles (endosomes) (Mellman *et al.*, 1986). However, the fact that *any* cytotoxicity is seen at all (comparison of conjugate B with conjugate D) indicates that *some* active (native) gelonin was indeed released from the conjugate, most likely in an acidic intracellular compartment. Whatever the explanation for the poor efficacy of this conjugate, these results with IL-2 conjugates suggested that gelonin is more readily released to the cytoplasm from a disulfide-linked conjugate than from a conjugate with an acid-labile linker.

In the present study, at least three factors may have affected the efficiency with which the IL-2–gelonin conjugates are internalized. Firstly, it is clear from binding studies that the attachment of gelonin to IL-2 decreases the affinity of the lymphokine for its receptor by about 1 order of magnitude. Such a decrease in binding affinity has been reported for a number of hormone–toxin constructs (Chang *et al.*, 1977; Bacha *et al.*, 1983) and may reflect the steric effects of attaching a protein toxin to a relatively small targeting vehicle, although we cannot rule out from these experiments an effect on binding due to the chemical modification itself. Secondly, although T lymphocytes express as many as 60 000 IL-2 receptors per cell, the efficient internalization of IL-2 occurs via a small population of high-affinity IL-2 receptors which are present on activated T-cells at a density of only 2000-4000 receptors per cell (Robb *et al.*, 1981; Weissman *et al.*, 1986). Thirdly, in the test system used, stimulation of lymphocytes by concanavalin A is known to result in the autocrine production of IL-2 (Robb *et al.*, 1981), and the endogenous IL-2 produced may reduce the cytotoxicity of IL-2–gelonin conjugates by direct competition at the level of the receptor. Clearly, these factors may limit the potential cytotoxicity of the IL-2–gelonin conjugates.

The cytotoxicity of the IL-2–gelonin made with a disulfide linker for cells expressing the IL-2 receptor was similar to that of an immunotoxin prepared using the A chain of ricin and an anti-IL-2 receptor antibody (anti-Tac) which binds to an epitope found in the p55 subunit of both the high- and low-affinity IL-2 receptors (Krönke *et al.*, 1986). However, these conjugates of single-chain ribosome-inactivating proteins targeting the IL-2 receptor, and other similar conjugates targeting other antigens, are far less potent than intact ricin (Goldmacher *et al.*, 1989; Blättler *et al.*, 1989). These observations have led to the suggestion that the B chain of ricin is not only responsible for binding to the cell surface but may also participate in the transport of the A chain across the membrane (Vitetta *et al.*, 1983; Blättler *et al.*, 1989) or may direct the A chain to a particular intracellular compartment from which the A chain can gain access to the cytoplasm (Goldmacher *et al.*, 1992; Newton *et al.*, 1992). Similar cell surface binding and transport functions have been ascribed to distinct portions of the bacterial toxins diphtheria toxin (Greenfield *et al.*, 1987) and pseudomonas exotoxin A (Hwang *et al.*, 1987). The selective removal or modification of the binding domains of these proteins by protein engineering has resulted in the generation of toxins (Greenfield *et al.*, 1987; Hwang *et al.*, 1987) which may be more potent than proteins such as gelonin or ricin A-chain if they retain all the elements important for translocation or correct intracellular routing (Pastan *et al.*, 1992). A number of genetic constructs have been reported in which such engineered proteins have been linked to hormones, and in particular, to IL-2 (Murphy *et al.*, 1988; Pastan *et al.*, 1992). These constructs appear to show selective toxicity both *in vitro* (Lorberboum-Galski *et al.*, 1988; Ogata *et al.*, 1988) and *in vivo* (Kelley *et al.*, 1988; Case *et al.*, 1989; Lorberboum-Galski *et al.*, 1989; Pastan *et al.*, 1992). Unfortunately, comparison of the potencies of conjugates of pseudomonas exotoxin A or diphtheria toxin with that of the IL-2–gelonin conjugate (disulfide link) is not possible owing to the wide variation in the biological test systems used in different laboratories.

An alternative approach to the production of a more potent toxin for specific targeting has been to chemically modify the cell-surface binding sites of ricin in order to block the ability of the toxin to bind to cells, while retaining the B-chain functions that promote efficient A-chain translocation into the cytoplasm (Lambert *et al.*, 1991a,b). It is likely that such modified toxins would also be highly effective reagents for the production of toxic conjugates using lymphokines such as IL-2.

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