Comparison of the Cytotoxic Effect of Hormonotoxins Prepared with the Use of Heterobifunctional Cross-Linking Agents *N*-Succinimidyl 3-(2-Pyridyldithio)propionate and *N*-Succinimidyl 6-[3-(2-Pyridyldithio)propionamido]hexanoate

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With the aim of developing cytotoxic hybrid molecules which can be selectively targeted to specific cells in the gonads, a single chain ribosome-inactivating protein, gelonin, was conjugated to ovine luteinizing hormone (oLH) with the use of heterobifunctional cross-linking agents *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and long-chain SPDP. Four hormonotoxins were synthesized having a variable spacer arm between oLH and gelonin. The spacer arms in C200A, C210A, C220A, and C230A were 13.6, 22.4, 22.4, and 31.2 Å long, respectively. Extensive physicochemical and biochemical analysis revealed a 1:1 molar ratio of the ingredients in its oLH–S–S–gelonin conjugates. The linkage occurred through the ϵ-NH_2 group of the α-subunit of oLH as judged from RP-HPLC analysis. The hormonotoxins retained substantial receptor binding ability, steriodogenic activity, and immunoreactivity of oLH and gelonin to their respective antibodies. Hormonotoxins bind to Leydig tumor cells via oLH, leaving gelonin free as judged by competitive displacement analysis. The hormonotoxins internalized to a sufficient degree to effectively inhibit protein synthesis. Upon comparison, immunoreactivity, receptor binding steroidogenic activity, and cytotoxicity of oLH–S–S–gelonin conjugates prepared with the use of only LC-SPDP (C230A, 31.2-Å spacer arm) and by using both SPDP and LC-SPDP (C210A and C220A, 22.4-Å spacer arm) were found to be comparable with that of conjugate prepared with SPDP alone (C200A, 13.6-Å spacer arm). Therefore, it may be concluded that the cytotoxicity of oLH-based hormonotoxin remained unaffected with the use of long-chain spacer arms which are believed to be used generally to avoid steric hindrance.

INTRODUCTION

The selective destruction of tumor cells by using hybrid molecules possessing both the functions of specific recognition and cytotoxic ability has been under active investigation (for review, see refs 1–5). These novel antitumor agents called "immunotoxins" have been synthesized in several laboratories by covalently linking the bacterial or plant toxins to the antibodies directed against the tumor-associated antigens (*1–5*). The conjugates, after binding to the antigens on the target cell surface, are endocytosed, and the toxin irreversibly inactivates the protein synthesis machinery of the cells (*1–5*). Among plant toxins, the most widely used are ricin, from castor bean, and abrin, from jequerity bean. These toxins are glycoproteins comprised of two chains: the B-chain, which binds to galactose residues on the cell surface, and the A-chain, which translocates in the cell and arrests protein synthesis machinery by enzymatically inactivating the ribosomes (*6–9*). The nonspecific toxicity of these toxins due to B-chain was avoided either by using immunotoxins in the presence of excess galactose or by completely depleting B-chain from the ricin. Thus the isolated A-chain was directly linked to the antibody molecule and the immunotoxin thus constructed was effective in a variety of animal models (*1–5*). Similar to the ricin A-chain, single-chain RIP[1] such as PAP, gelonin, and saporin are nontoxic to the intact cell but can inactivate the eukaryotic ribosomes and inhibit protein synthesis once they gain entry into the cell by a receptor-mediated process (*6–9*).

Like immunoglobulins, several hormones or growth factors such as human placental lactogen (*10*), insulin (*11*), human chorionic gonadotropin (*12, 13*), thyrotropin-releasing hormone (*14*), ovine luteinizing hormone (*15*), and corticotropin-releasing factor (*16*) have been conjugated to selected toxins. Recently, with the aim of targeting such toxins to the selected cells in the gonads, we have designed bioeffective conjugates of ovine LH with a single chain RIP, gelonin, by using SPDP as a cross-linking agent (*15, 17–21*). In these studies, both proteins

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[1] Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; RIP, ribosome-inactivating protein; oLH, ovine luteinizing hormone; PAP, pokeweed antiviral protein; FCA, Freund's complete adjuvant; IFA, incomplete Freund's adjuvant; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; LC-SPDP, *N*-succinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate; Iodogen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; CMC, (carboxymethyl)cellulose, RP-HPLC, reverse-phase high-performance liquid chromatography, RPMI, Roswell Park Memorial Institute, SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, TCA, trichloroacetic acid, TFA, trifluoroacetic acid, EDTA, ethylenediaminetetraacetic acid, RIA, radioimmunoassay, eCG, equine chorionic gonadotropin, hCG, human chorionic gonadotropin, BMV, Brome mosaic virus, mRNA, messenger ribonucleic acid, GnRH, gonadotropin releasing hormone, DMEM, Dulbecco's modified Eagle's medium, HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid).

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were thiolated by SPDP and subsequently reacted under appropriate conditions to form an oLH–S–S–gelonin complex (*15*, *17–21*). SPDP modification introduces a 6.8-Å spacer arm, which may induce some steric hindrance in the conjugates for the receptor recognition. In order to limit such steric hindrance, recently a long-chain SPDP has been introduced which has a 15.6-Å spacer arm. The present paper describes a comparative study of the synthesis, characterization, and bioefficacy of oLH–gelonin conjugate by using SPDP and LC-SPDP as heterobifunctional cross-linking agents.

EXPERIMENTAL PROCEDURES

Heterobifunctional cross-linking reagents (SPDP and LC-SPDP) and DTT were obtained from Pierce, Rockford IL. Sephadex G-25, G-100 and CMC-52 were from Pharmacia Fine, Chemicals, Sweden. BSA (Cohn fraction IV), FCA, and IFA were obtained from Sigma, Chemicals, St. Louis, MO. Radioactive Iodine ($Na^{125}I$, IMS-30) was obtained from Amersham, Corp. L-(3,4,5-^{3}H)leucine of specific activity 143 Ci/mmol was purchased from Dupont–NEN. Dry seeds of *Gelonium multiflorum* were purchased from United Chemicals and Allied Products, Calcutta, India. All other chemicals were of highest purity available commercially.

oLH was purified from the sheep pituitary powder according to the procedure described earlier (*15*, *22*). The purified hormone was subjected to physicochemical, immunological, and biological characterization especially for receptor binding and steroidogenic activity prior to use for covalent coupling to gelonin. The hormone was stored as a dry powder at 4 °C.

Gelonin was isolated from the dry deshelled seeds of *G. multiflorum* using aqueous extraction, ammonium sulfate precipitation, cation-exchange, and finally gel-filtration chromatography (*23*, *24*). A recent comparative study (*25*) on three methods of purification of gelonin clearly revealed that the presently used method yields more purified preparation, devoid of nonproteinous materials which absorb at 280 nm (*25*). The purified protein was subjected to purity, homogeneity (by using RP-HPLC), and molecular weight determinations (by a SDS–PAGE technique). Gelonin used in the present investigation was judged to be over 98% pure, as analyzed by RP-HPLC, and retained its ability to inhibit protein synthesis in an *in vitro* cell-free translation system (*26*).

Polyclonal antibodies against oLH and gelonin were developed in rabbit by immunizing them following the protocol described earlier (*15*, *24*). Sera were collected after the appropriate booster injections, and the immunoglobulin fraction was obtained after ammonium sulfate precipitation (*15*, *24*). The immunoglobulin fractions were dialyzed extensively, lyophilized, and stored as powder at 4 °C.

Murine Leydig tumor cell line, MLTC-1, used in the present investigation was gifted by Dr. R. Victor Rebois, NIH, Bethesda, MD. The cells were propagated and maintained in culture according to the published procedure (*27*). The cells grown in 24-multiwell plates were used to determine steroidogenic and cytotoxic activity of the hormonotoxins.

Modification of ϵ-NH_2 Groups of oLH/Gelonin with SPDP or LC-SPDP. The ϵ-NH_2 groups of oLH or gelonin were modified by LC-SPDP according to the standard procedure described for SPDP (*28*). Briefly, the protein was allowed to react with cross-linking agents in an appropriate molar ratio to modify one ϵ-NH_2 group ($\sim$1:1.2 ± 0.3) in each protein. oLH/gelonin dissolved in sodium phosphate buffer (0.1 M, pH 7.5, containing 0.15 M NaCl) was activated with SPDP or LC-SPDP, dissolved in the minimal volume of ethanol. The reaction was carried out at 25 °C for 30 min and then passed through a G-25 column and eluted with 5 mM ammonium bicarbonate. The protein peak eluting in the void volume of the column was pooled, lyophilized, and stored at 4 °C for further analysis. The extent of amino group modification was determined on the basis of liberation of pyridine-2-thione upon DTT treatment. The reaction was followed by recording the UV–visible spectra. From the absorption at 343 nm, the number of pyridine-2-thione groups liberated was calculated using the extinction coefficient $\epsilon_{343} = 8.08 \times 10^{-3}\ M^{-1}$ (*29*). The absorption at 280 nm was corrected for pyridine-2-thione contribution (*29*).

To initiate conjugation, the SPDP- or LC-SPDP-modified gelonin in 0.1 M sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl was treated with DTT to generate an SH group and then gelonin–SH was passed through a Sephadex G-25 column to remove small molecular weight reduced products. Prior to G-25 chromatography, the UV–visible spectra was recorded in order to determine the number of SH groups generated as described above. Gelonin–SH was then mixed with oLH-SPDP or –LC-SPDP in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl. The mixture was left at 25 °C for 24 h, later concentrated by Centriprep-10, and fractionated on a G-100 column at 4 °C using 0.05 M ammonium bicarbonate buffer. Prior to G-100 chromatography, the UV–visible spectra was recorded to determine the liberation of pyridine-2-thione during the conjugation reaction (see the Results and Discussion section). The contribution of pyridine-2-thione absorption at 280 nm was corrected in calculating its molar concentration and the hormone:gelonin molar ratio was determined. Appropriate conjugated fractions were pooled, dialyzed, and lyophilized.

Characterization of Hormonotoxins. The conjugates of oLH with gelonin as prepared above were subjected to extensive physicochemical, immunochemical, and biological characterization. SDS–PAGE and RP-HPLC analysis were carried out to establish the purity and molecular weight of the conjugate.

RP-HPLC analysis. RP-HPLC analysis was used to analyze the purity of the ingredients and hormonotoxins as described above. RP-HPLC was performed on a Water's μBondapak phenyl column (4 mm × 250 mm) using a continuous gradient as described (*15*, *17–19*, *24–28*). The samples dissolved in water containing 0.1% TFA (solvent A) were run on the above column pre-equilibrated in solvent A + 25% CH_3CN containing 0.1% TFA. A gradient of 25–50% was run over 30 min at a flow rate of 2 mL/min in a DuPont HPLC, and data were analyzed by a computer.

Immunoreactivity determination. The immunoreactivity of oLH, gelonin, their SPDP- and LC-SPDP-modified derivatives and hormonotoxins was determined by a competitive displacement analysis as described earlier (*15*, *17–19*, *24–28*). ^{125}I–oLH required for RIA was radioiodinated by the Iodogen method (*30*) essentially following the procedure described for GnRH (*31–33*). Free $Na^{125}I$ and ^{125}I–oLH (or ^{125}I–gelonin were separated by gel-filtration chromatography on a Sephadex G-25 (1.0 × 50 cm) column. The specific activity as determined by an autodisplacement method was in the range of 50–80 μCi/μg. The labeled hormone/gelonin was stored in aliquots at −70 °C and used within a month of preparation.

The competitive displacement assay of the hormone

was performed essentially following the procedure described earlier (*15*, *17–19*, *28*). Briefly, the test samples, labeled and unlabeled oLH, and anti-oLH antibody were diluted in assay buffer (0.05 M sodium phosphate, pH 7.5, containing 0.15 M NaCl and 0.1% each of BSA and sodium azide). One hundred microliter of diluted anti-oLH antibody giving 30–50% binding in the same buffer was incubated with 100 μL of different concentrations of test samples (competitors). After 12 h of incubation at 4 °C, 100 μL of ^{125}I–oLH was added and further incubation for 12 h at 4 °C was carried out. Bound and unbound ^{125}I–oLH were separated by addition of 0.2 mL of 1:5 diluted Pansorbin (Calibiochem). The tubes were incubated further at room temperature for 20–30 min and centrifuged at 3000*g* for 15 min. The supernatant containing unbound ^{125}I-oLH was removed by aspiration and the pellet was counted in a LKB-Rack mini γ counter. The inhibition lines were obtained by plotting $(B/B_0) \times 100$ against the log dose of the competitor (where B_0 represent the binding of ^{125}I–oLH in the absence of cold oLH and B in the presence of competitor). The extent of percentage cross-reactivity was expressed as $C = (L_{50}/U_{50}) \times 100$, where the L_{50} is the dose of the competitor which shows 50% inhibition and U_{50} is the dose of native oLH which shows the 50% inhibition (*15*, *17–20*).

Immunoreactivity of gelonin was also determined by a competitive displacement assay as described earlier (*24*, *25*) with minor modification. The radioiodination of gelonin was carried out by the Iodogen method as described for oLH. Five micrograms of gelonin was radioiodinated with 1 mCi of Na^{125}I, and iodinated gelonin was purified on a Bio-Gel P6DG disposable column. The specific activity of the ^{125}I–gelonin, calculated by the incorporation efficiency, was ~80 μCi/μg. The immunoreactivity of the labeled gelonin was estimated by measuring the binding ability (>80%) in the presence of excess of anti-gelonin antibody (*24*, *25*).

Antibody binding and competitive binding assays were carried out following the methods described earlier (*24*, *25*) with the difference that double antibody precipitation was used instead of precipitation by Pansorbin. The method of calculation of the extent of cross-reactivity was the same as described earlier (*24*, *25*).

Receptor Binding Property. The receptor binding of oLH, SPDP-/LC-SPDP-modified oLH, and their conjugates with gelonin was determined by RRA using rat testicular homogenate as a source of gonadotropin receptors as described earlier (*15*, *17–19*). In brief, 100 μL each of labeled oLH (~70 000 CPM/tube), test samples, unlabeled oLH, and assay buffer (25 mM, Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 1 mg/mL BSA) were incubated at 22–25 °C for 16–20 h. The reaction was terminated by addition of 2 mL of assay buffer and centrifugation in cold at 3000*g* for 30 min. Supernatant was aspirated and the pellet was counted in a γ counter as described above.

Steroidogenic Ability Determination. The progesterone induction ability of oLH, SPDP- or LC-SPDP-modified oLH, and their conjugates to gelonin was evaluated by incubation with ovarian granulosa cells of immature female rats as described earlier (*15*, *17–19*). The immature female rats were primed with eCG and after 48-h incubation, the granulosa cells were collected and incubation with test samples in DMEM, containing 20 mM HEPES, pH 7.5, and 0.1% BSA at 37 °C under 95% oxygen and 5% carbon dioxide for 4 h. Progesterone secreted in the medium was estimated by a specific RIA as reported earlier (*15*, *17–19*).

In-Vitro Cell Free Translation Assay. The inhibitory activity of gelonin and their SPDP-modified derivatives on cell-free protein synthesis was determined using a system consisting of nuclease-treated rabbit reticulocyte lysate (*34*, *35*). The extent of protein synthesis was determined by measuring radioactive leucine incorporated into the TCA-insoluble protein fraction. Briefly, in a total of 15 μL of reaction mixture, 2 μL of test sample was incubated with 1 μL of BMV mRNA (0.5 μg/μL), 2 μL each amino acid mixture (minus leucine) and tritiated leucine, 0.5 μL each of 500mM potassium acetate and 200mM magnesium acetate, and 7 μL nuclease treated rabbit reticulocyte lysate. The positive control was incubated with mRNA (no test sample) while the negative control received an equal volume of RNAse-free water. After incubation at 30 °C for 60 min, 5 μL of reaction mixture was spotted at least 10–15 places on the 3-mm Whatman filter paper and dipped in cold 5% TCA containing 0.2% leucine. Three more washings of 2 min each were carried out with cold TCA. The paper was heated at 90 °C in 5% TCA for 2 min and then quickly washed with cold ethanol and dried in the air. The protein precipitated was counted in a scintillation counter (Beckman of ~70% efficiency) after adding 8 mL of scintillation cocktail. Percentage of protein synthesis was calculated on the basis of radioactivity incorporated in the precipitate. The protein synthesis inhibition = $(B/B_0) \times 100$ where B is the radioactivity incorporated in the TCA precipitate in the presence of inhibitor and B_0 is in the absence of any inhibitor. IC_{50} is the dose of inhibitor required for 50% inhibition of protein synthesis.

Binding and Cytotoxicity to Leydig Tumor Cells. The binding and cytotoxicity experiments were carried out as desribed earlier (*17–19*). Briefly, for binding experiments, MLTC-1 cells (~1.5×10^5 cells/well) were plated in 24-well plates (flat bottom, Flow laboratory) and grown for 24–48 h. When the cells occupied most of the space, the wells were washed two times with serum-free medium. The cells received different concentrations of hormonotoxins and appropriate concentration of gelonin as a control. The plates were incubated for 2–4 h at 37 °C in a humidified chamber containing 95% O_2 and 5% CO_2. After incubation, the wells were washed three times with serum-free medium and each well received 50 μg of gelonin antibody purified by 50% ammonium sulfate fractionation as described above. After 2-h incubation, the wells were again washed with serum-free medium and incubated with ^{125}I–protein-A (~200 000 CPM/well) for 2 h. The iodination of protein-A was carried out by the Iodogen method (*30*) essentially as described above for oLH. The wells were washed extensively, and the bound radioactivity was counted after solubilizing cells with 0.1 N NaOH. For competition experiments, 10 μg/well concentration of the conjugate was used. This was determined after preliminary binding experiments. Fifty micrograms/well of native oLH was added together with the conjugate. After 2-h incubation in the presence of native oLH, the wells were again washed with serum-free medium and the conjugate bound to the cells was estimated by addition of ^{125}I–protein-A as described above.

The test for cytotoxicity of the conjugate was carried out on MLTC-1 cells as described before (*17–19*). Briefly, the cells were plated in RPMI-1640 medium in 24-multiwell plates, and after 24-h culture, the medium was replaced with the fresh medium, different concentrations of test samples were added, and plate was left for 18–24 h more at 37 °C in a humidified chamber containing 95% O_2 and 5% CO_2. The cells were washed after 24 h with serum-

Table I. Conjugates Synthesized with the Use of SPDP and LC-SPDP

oLH–gelonin conjugate code	spacer arm (Å)	cross-linking agent used: activation of oLH with	cross-linking agent used: activation of gelonin with	structure
C200A	13.6	SPDP	SPDP	oLH–HNC(O)$(CH_2)_2SS(CH_2)_2$C(O)NH–gelonin
C210A	22.4	SPDP	LC-SPDP	oLH–HNC(O)$(CH_2)_2SS(CH_2)_2$C(O)NH$(CH_2)_5$C(O)NH–gelonin
C220A	22.4	LC-SPDP	SPDP	oLH–HNC(O)$(CH_2)_5$HNC(O)$(CH_2)_2SS(CH_2)_2$C(O)NH–gelonin
C230A	31.2	LC-SPDP	LC-SPDP	oLH–HNC(O)$(CH_2)_5$HNC(O)$(CH_2)_2SS(CH_2)_2$C(O)NH$(CH_2)_5$C(O)NH–gelonin

free medium and were pulsed for 2 h with [^{3}H]leucine (0.5 μCi/well) in leucine-free RPMI-1640 medium. After 4-h incubation, the cells were extensively washed with 5% TCA to remove nonspecifically bound radioactivity. The cells were solubilized with 0.5 mL of warm 0.1 N NaOH, and solubilized protein was mixed with 5 mL of scintillation fluid and counted in the scintillation counter as described above.

RESULTS

A careful titration to determine the necessary molar ratio of oLH/gelonin versus SPDP or LC-SPDP was carried out. Present and earlier experience with SPDP (*24*, *25*, *28*) revealed that a molar ratio of 1:2 for oLH/gelonin: SPDP thiolated 1.2 ± 0.2 amino groups. Similarly, our recent investigation on the effect of LC-SPDP modification of ϵ-NH_2 groups of oLH clearly revealed that a 1:2 molar ratio of oLH and LC-SPDP thiolated 1:1.3 ± 0.2 amino groups per molecule (*36*). The conjugation of oLH to gelonin was initiated following the protocol as described in the Experimental Procedures. The conjugation mixtures were fractionated on a Sephadex G-100 column and elution profiles of all the conjugates are shown in Figure 1. The upper portion of Figure 1A shows the elution positions of oLH, gelonin, and the void volume (V_0) of the column. The chemical structures of four different conjugates, C200A, C210A, C220A, and C230A are shown in Table I. This table also shows the activation of oLH and gelonin with SPDP and LC-SPDP. In all the cases, SPDP-/LC-SPDP-modified gelonin was only treated with DTT to generate SH groups which were subsequently used for conjugation with oLH/SPDP or LC-SPDP. In C200A conjugate both the ingredients were activated with SPDP and later the conjugation was initiated after mixing with the DTT-treated gelonin–SPDP as described earlier (*15*, *17–19*). C210A and C220A conjugates were prepared with the use of both SPDP and LC-SPDP while C230A was synthesized with the use of only LC-SPDP. Therefore the spacer arm in C200A conjugate was calculated to be 13.3 Å long, while in C230A the spacer arm was 31.2 Å long. The C210A and C220A conjugates had spacer arms of 22.4 Å in length (Table I). The protein peak appearing before oLH and gelonin was considered to be their conjugate and was pooled (designated as A peak). The unconjugated oLH or gelonin peaks were designated as B and C, respectively. In each conjugate, the pool A peak was concentrated and rerun on the same column (Figure 1). In the rechromatographic run, the major peak eluting before oLH was pooled (designated as ARA) and lyophilized directly. The unconjugated peaks of oLH and gelonin were lyophilized separately. Figure 1A shows the elution profile of C200 and C210 conjugate whereas Figure 1B recorded the profile of C220 and C230 conjugation mixture. A small peak appearing between fractions 110 and 140 was due to the release of pyridine-2-thione, which appart from its absorption at 343 nm also absorbs at 280 nm. Therefore, in order to determine the molar concentration, the molar extinction coefficient of pyridine-2-thione is used to apply a correction for its contribution at 280 nm (*29*).

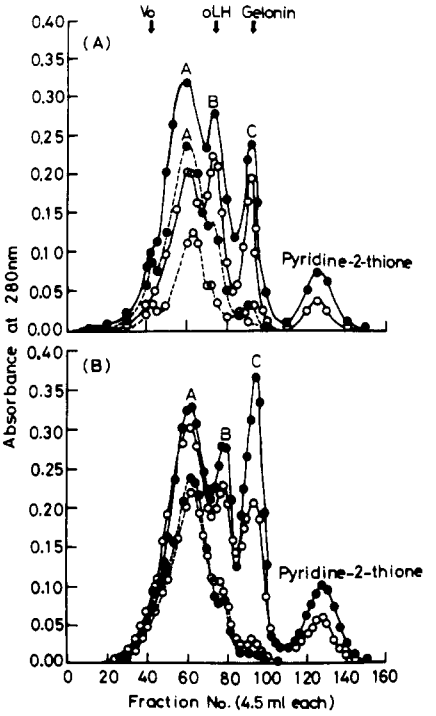

Figure 1. Gel-filtration chromatography of oLH–gelonin conjugation mixtures on a Sephades-G-100 column. The protein was eluted with 50 mM ammonium bicarbonate at a flow rate of 20 mL/h. Arrows in the upper portion of A indicate the void volume and elution positions of oLH and gelonin. (A) This shows gel-filtration chromatograms of the conjugation mixtures of C200 (●) and C210 (○). The major peak eluting before oLH and gelonin was their conjugate (designated as C200A and C210A). This peak was concentrated and rechromatographed on the same column. The dashed line (– –) chromatograms show the rechromatographed C200A and C210A. The peak A (designated as C200ARA and C210ARA) was pooled and lyophilized directly. The unconjugated peaks of oLH and gelonin were lyophilized separately. (B) Gel-filtration chromatograms of the conjugation mixtures of C220 (●—●) and C230 (○—○). The chromatograms shown by ●– –● and ○– –○ indicate rechromatography of peak A of peak C220 (as C220A) and C230 (as C230A). The major peaks of the rechromatography of C220A and C230A were designated as C220ARA and C230ARA, respectively. As described above, these portions of the peaks were directly lyophilized.

In SDS–PAGE analysis, the patterns of the conjugates were compared with those of the ingredients, oLH and gelonin. The samples analyzed on SDS–PAGE under reduced and unreduced conditions are shown in Figure 2. Under nonreduced conditions (Figure 2, left panel), the conjugates showed the absence of either free oLH or gelonin (lanes 3, 4, 7, 8, and 11). These conjugates showed the distinct presence of both oLH and gelonin once they are reduced (Figure 2, right panel). The native oLH appears as a diffused band between 31 and 45 kDa (Figure 2, left panel, lane 13) and gelonin appears as a sharp band at ~31 kDa (lane 12, left and right panel). The conjugates showed multiple bands of slower migrating component with apparent molecular weights of 55–116 kDa (lanes 3, 4, 7, 8, and 11). The conjugates showed the distinct

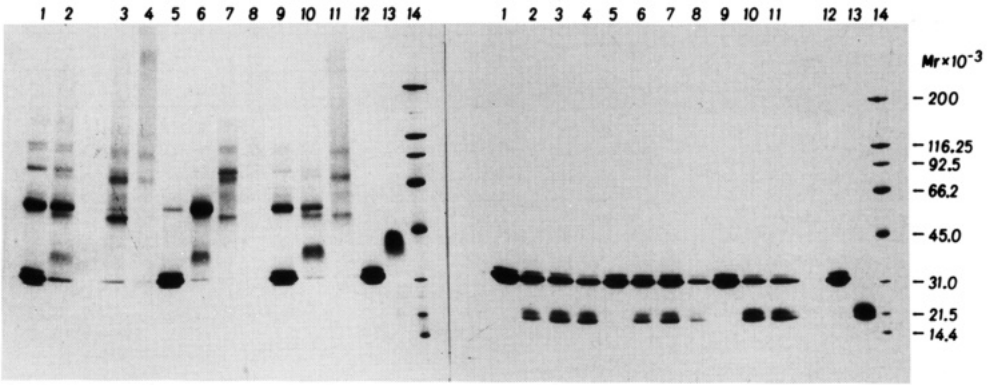

Figure 2. SDS–PAGE analysis of oLH–gelonin conjugate prepared by using SPDP and LC-SPDP. SDS–PAGE (reduced, right panel; nonreducing, left panel) analysis of the conjugate and other peaks. A gel consisting of 5–15% (w/v) acrylamide was used. The gel was fixed in methanol/acetic acid, stained with Commassie Blue and destained with methanol/acetic acid for 24 h. Lane 1, C210C-P85-100; lane 2, C210B-P72-80; lane 3, C210A-P50-65; lane 4, C210ARA-P50-67; lane 5, C220C-P87-100; lane 6, C220B-P72-80; lane 7, C220ARA-P50-66; lane 8, C200A-P35-46; lane 9, C230C-P87-100; lane 10, C230B-P75-80; lane 11, C230ARA-P45-65; lane 12, gelonin; lane 13, oLH; lane 14, molecular weight markers [myosin (200 kDa); β-galactosidase (116.25 kDa), phosphoamylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), Soyabean trypsin inhibitor, (21.5 kDa), lysozyme, (14.4 kDa)]. A sample of 40 μg each was run under reduced (right) and unreduced (left) panel conditions on the gel. Densitometric scan and quantitation were done on the reduced gel in order to determine molar ratio of the conjugate.

presence of both oLH and gelonin once they are reduced. (Figure 2, right panel).

The conjugates synthesized above were further analyzed by RP-HPLC. Figure 3 shows the RP-HPLC chromatograms of native oLH, gelonin, and their SPDP- and LC-SPDP-modified derivatives. The analysis was carried out on the Water's μBondapak phenyl column, using a continuous gradient (as described in the legend of the Figure 3). A comparative study of oLH and SPDP- or LC-SPDP-modified oLH chromatograms clearly reveal that ϵ-NH_2 group modification primarily occurs in the α-subunit of the hormone as seen by the emergence of new prominent fractions at t_R = 8.2 and 9.4 min for SPDP–oLH and t_R = 8.98, 9.4, and 9.98 min for LC-SPDP–oLH (Figure 3A). In both cases, the pattern of LH-β peaks in the elution zone at 14–16 min remains essentially unaltered (Figure 3A). The appearance of more peaks at the higher retention time may be due to the increase in hydrophobicity of LC-SPDP compared to SPDP. The elution pattern of SPDP– or LC-SPDP–gelonin essentially remains the same at t_R ~21.10 min, except that its extinction coefficient is higher. RP-HPLC of different conjugates are also shown in Figure 3B–D. A comparison of RP-HPLC chromatograms clearly reveals the presence of the LHβ component in the expected zone, but there is a complete absence of the modified α-subunit, as this now remains covalently linked to gelonin in a disulfide bond. This further confirms that the conjugation selectively occurs through one of the amino groups of the α-oLH. The acidic conditions used in the RP-HPLC causes complete dissociation of the oLH subunits, which is distinctly separated by the gradient elution. The β-subunit eluting as a doublet in the t_R = 14–16 min zone was due to the αLH–S–S–gelonin complex. Due to the higher extinction coefficient of gelonin, the conjugate peak eluting at t_R = 21–23 min as a broad peak indicated a relatively higher concentration than for the β-subunit (Figure 3B–D). Furthermore, the conjugates do not have free hormone contamination (free SPDP- or LC-SPDP-modified oLH) because of the lack of α-subunit peaks, which if present would have been easily detectable under the conditions employed here. This observation further confirms the SDS–PAGE data in which the conjugates did not show the presence of either of the ingredients (Figure 2 left panel, lane 3, 4, 7, 8, and 11).

The molar ratio of oLH and gelonin in the conjugates

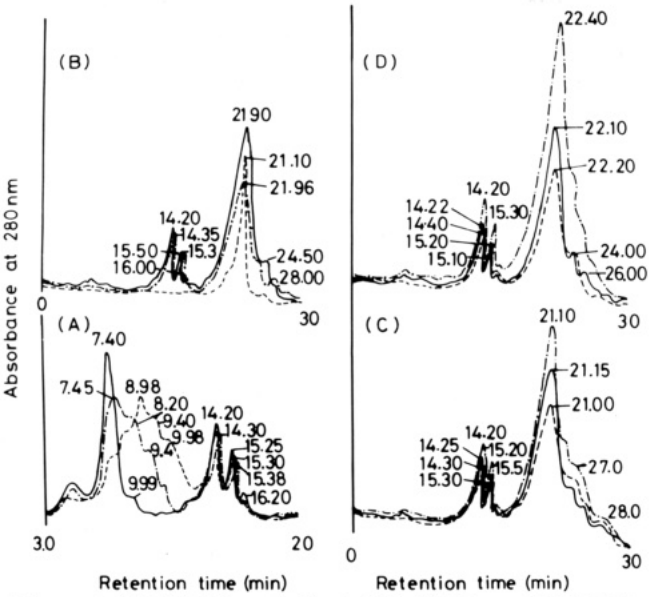

Figure 3. RP-HPLC analysis of oLH, SPDP- and LC-SPDP-modified oLH, gelonin, and the conjugates. The samples were dissolved in H_2O containing 0.1% TFA (solvent A) and the analysis was performed on a Water's μBondapak phenyl column (4 × 250mm) and equilibrated in solvent A + 25% CH_3CN and 0.1% TFA. A gradient of 25–50% was run over 30 min at a flow rate of 2 mL/min and monitored at 280 nm. In case of oLH, oLH–SPDP, and oLH–LC-SPDP, the chromatograms were analyzed between 3 and 20 min; however in case of gelonin, oLH–gelonin conjugates the chromatograms were analysed between 0 and 30 min. The RP-HPLC chromatograms of oLH (—), oLH–SPDP (-·-) and oLH–LC-SPDP (- -) are shown in pane A. Please note that the modification occurs in α-subunit (t_R = 7.4 min) while β-subunit (t_R = 14–16 min) remains unaltered. The RP-HPLC chromatograms of gelonin-SPDP (- -) and oLH-gelonin conjugates C200A (—) and C200ARA (-·-) are shown in panel B. The RP-HPLC chromatograms of C210A (—), C210ARA (- -) and C220A (-·-) are shown in panel C. The RP-HPLC chromatograms of C220ARA (—), C230A (- -) and C230ARA (-·-) are shown in panel D. The structure of C200, C210, C220, and C230 conjugates is shown in Table I. Note all the conjugates did not show the presence of α-subunit as it was covalently conjugated to gelonin. Under the acidic conditions, the β-subunit, which is associated noncovalently, was dissociated from the complex and eluted around t_R = 14–16 min.

was determined by spectrophotometric, SDS–PAGE, and amino acid analysis as described earlier (*15*, *17–19*). Spectrophotometrically, the molar concentration of py-

Table II. Amino Acid Composition of oLH, Gelonin, and Their Conjugates Prepared with the Use of SPDP and LC-SPDP[a]

amino acid	molar ratio oLH	molar ratio gelonin	for 1:1 adduct	experimental observation for the conjugates C200ARA	C210ARA	C220ARA	C230ARA
Lys	12	20	32	30.4	31.4	30.2	32.5
His	6	2	8	8	8	8	8
Arg	11	11	22	23.0	22.4	21.9	22.4
Asp	11	26	37	37.5	38.2	34.4	37.6
Thr	16	12	28	24.5	26.5	28.5	29.9
Ser	14	12	26	25.5	24.5	26.8	27.0
Glu	14	22	36	37.5	38.2	38.0	38.0
Pro	27	10	37	ND	ND	ND	ND
Gly	11	15	26	26.4	26.2	28.0	28.4
Ala	15	15	30	32.4	30.5	33.0	31.4
half Cys	22	2	24	ND	ND	ND	ND
Val	13	13	26	25.2	25.8	27.0	26.8
Met	7	2	9	ND	ND	ND	ND
Ile	7	14	21	20.4	21.5	19.8	21.9
Leu	14	20	34	31.5	35.8	36.0	34.5
Tyr	7	9	16	16.2	17.4	18.0	16.9
Phe	8	11	19	19.9	20.4	22.0	21.5
Trp	0	?	?	ND	ND	ND	ND
carbohydrate	+	+	+	+	+	+	+

[a] oLH values are from the structure of the α- and β-subunit. Gelonin composition was determined on the purified preparation used for the conjugation in the present investigation. ND, not determined.

Table III. Molar Ratio of oLH and Gelonin in the Conjugates As Determined by Different Methods

conjugate code	molar ratio (oLH:gelonin) spectrophotometric	gel electrophoresis	amino acid composition
C200ARA	1:1.30	1:1.20	1:1
C210ARA	1:1.40	1:1.32	1:1
C220ARA	1:1.20	1:1.20	1:1
C230ARA	1:1.30	1:1.25	1:1

ridine-2-thione liberated after DTT treatment of the SPDP-/LC-SPDP-gelonin followed by mixing with SPDP-/LC-SPDP-oLH was estimated (*15*, *17–19*). In order to determine the ratio in the conjugates by SDS–PAGE, different concentrations of oLH and gelonin were run under reducing conditions and a concentration dependence of the staining pattern was established by densitometry (*17*). A fixed amount (40 μg) of the conjugate was run in a parallel gel. The protein bands were stained with Coomassie Brilliant Blue stain and scanned for protein concentration. The amino acid composition of oLH, gelonin, and their conjugates was determined by the ninhydrin method as described (*15*, *18*) and are recorded in Table II. The experimentlly determined amino acid composition of the conjugates closely agrees with the theoretical prediction on the basis of a 1:1 ratio (Table III). The molar ratio of oLH and gelonin in the conjugate thus determined are recorded in Table III. Reactivity of oLH and gelonin with their specific antibodies in the different fractions of the conjugates was determined by competitive RIA as described in the Experimental Procedures. The competitive displacement curves of oLH is shown in Figure 4. The quantitative immunoreactivity data of oLH and gelonin are recorded in Table IV. The immunoreactivity of oLH was drastically reduced (>86% activity) after a single ϵ-NH_2 group modification by SPDP. In case of LC-SPDP-modified oLH, the activity was further reduced (>89%) (Figure 4, Table IV), which may be due to the increase in hydrophobicity of oLH–LC-SPDP. However, after conjugation, with gelonin, the oLH immunoreactivity was further affected by 3–5% (Table IV). When oLH–SPDP or oLH–LC-SPDP immunoreactivity was taken as 100%, the activity of the gelonin conjugates varied from 22 to 41% (Table IV). In a 1:1 molar ratio conjugate, the immunoreactivity was doubled (Table IV). Similarly, the gelonin immunoreactivity was reduced up

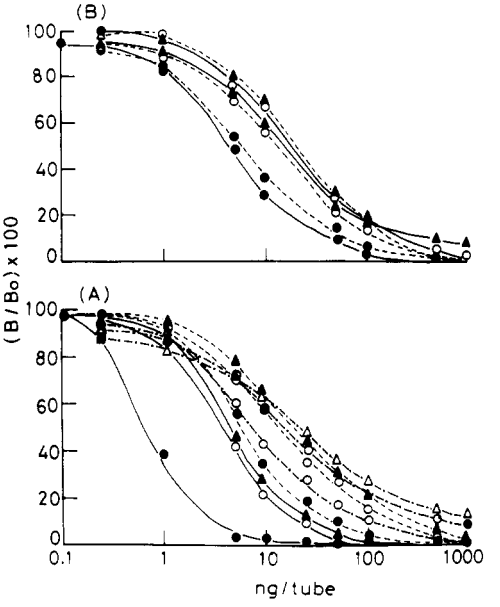

Figure 4. Immunological cross-reactivity of oLH antibody to oLH, its SPDP- and LC-SPDP-modified derivatives, and their conjugates to gelonin. The percent immunoreactivity was determined by competitive displacement analysis using specific RIA as described in the Experimental Procedures: Lower panel, A. The displacement cures are oLH (●—●), oLH-SPDP (○—○), oLH-LC-SPDP (△—△), C200A (▲- -▲), C200B (●- -●), C200ARA (○- -○), C230A (●- -●), C230B (○-·-○), C230ARA (△-·-△); upper panel, B, the displacement curves of C210A (○—○), C210B (●—●), C210ARA (▲—▲), C220A (○- -○), C220B (●- -●), and C220ARA (▲- -▲). The quantitative data based on these displacement curves are recorded in Table IV.

to 30–36% after a single ϵ-NH_2 group modification with SPDP or LC-SPDP. However, the activity was not further affected after conjugation with oLH (Table IV).

One of the primary requirements of biological activity of hormonotoxins are their binding to the hormone receptor. The receptor binding was determined by using rat testicular homogenate as a source of gonadotropin receptor. After a single NH_2 group modification with SPDP or LC-SPDP, the activity was reduced to 8.5% and 7.0%, respectively (Table V). This was further reduced upon conjugation to gelonin (Table V). As compared to SPDP–oLH or LC-SPDP–oLH with a single amino group modification and correction for increase in the molecular

Table IV. Immunoreactivity of oLH and Gelonin in the Conjugates[a]

hormone–cross-link–gelonin	hormone assay			gelonin assay		
		% cross-reactivity			% cross-reactivity	
	ED_{50} (ng)	A	B	ED_{50} (ng)	A	B
oLH	0.60	100	–			
oLH–SPDP	4.15	14.5	100			
oLH–LC-SPDP	5.45	11.0	100			
gelonin				0.90	100	
gelonin–SPDP				2.50	36.0	100
gelonin–LC-SPDP				3.00	30.0	100
oLH–S–S–gelonin						
C200A	18.70	3.2 (6.4)	22.10 (44.20)	5.45	16.50 (33.0)	45.80 (91.60)
C200B	3.75	16.0	110.6	18.00	5.00	13.90
C200C	ND	ND	ND	2.30	39.00	108.00
C200ARA	12.50	4.8	33.20 (66.40)	5.15	17.50	48.50 (97.00)
C210A-P50-65	51.80	3.8 (7.6)	26.26 (56.53)	5.00	18.00 (36.00)	60.00 (120.00)
C210B-P72-80	4.10	14.5	101.20	30.00	3.00	10.00
C210C-P85-100	ND	ND	ND	2.80	32.00	107.00
C210ARA-P50-67	14.30	4.20	29.00 (58.00)	5.50	16.50 (33.00)	54.54 (109.10)
C220A-P50-64	13.30	4.50 (9.00)	40.90 (81.80)	4.73	19.00 (38.00)	52.85 (105.70)
C220B-P72-80	6.00	10.00	90.83	30.00	3.00	8.33
C220C-P87-100	ND	ND	ND	2.25	40.00	111.11
C220ARA-P50-66	17.10	3.50 (7.00)	31.87 (63.74)	5.62	16.00 (32.00)	48.48 (88.96)
C230A-P52-65	15.80	3.80 (7.60)	34.50 (69.00)	5.62	16.00 (32.00)	53.40 (106.80)
C230B-P75-80	7.50	8.00	72.66	45.00	2.00	6.60
C230C-P87-100	ND	ND	ND	2.50	36.00	120.00
C230ARA-P45-65	18.75	3.20 (6.40)	29.06 (58.13)	5.60	16.00 (32.00)	53.60 (107.20)

[a] The immunoreactivity is expressed as a percentage with respect to native oLH or gelonin (column A) and oLH–SPDP/LC-SPDP or gelonin–SPDP or gelonin–LC-SPDP (column B), which were taken as 100%. The details of the experimental design are given in the Experimental Procedures. Numbers in parentheses for the hormone assay show values when corrected for molecular weight of the conjugate assuming 1:1 complex formation. In the gelonin assay similar correction also applies for the presence of hormone in the concerned fraction.

Table V. Receptor Binding Activity, Ribosome-Inactivating Property, and Steroidogenic Activity of oLH–S–S–Gelonin Conjugates[a]

hormone–cross-link–gelonin	receptor binding activity			protein-synthesis-inhibitor activity			steroidogenic activity (%)
		% activity			% activity		
	ED_{50} (ng)	A	B	IC_{50} (ng)	A	B	
oLH	0.95	100					100
oLH–SPDP	11.20	8.5	100				110–120
oLH–LC-SPDP	13.60	7.0	100				120–130
gelonin				0.25	100		
gelonin–SPDP				1.67	15.0	100	
gelonin–LC-SPDP				2.08	12.0	100	
oLH–S–S–gelonin							
C200A	79.10	1.20 (2.40)	14.16 (28.32)	5.43	4.6 (9.2)	30.75 (61.50)	3.4 (6.8)
C200ARA	59.40	1.60 (3.20)	18.85 (37.70)	4.80	5.2 (10.4)	34.80 (69.60)	3.2 (6.4)
C210A	68.00	1.40 (2.80)	16.48 (32.96)	6.25	4.0 (8.0)	33.30 (66.60)	4.0 (8.0)
C210ARA	61.00	1.56 (3.12)	18.36 (36.72)	5.70	4.4 (8.8)	36.50 (73.00)	3.6 (7.2)
C220A	105.00	0.90 (1.80)	12.95 (25.95)	4.50	5.5 (11.0)	37.10 (74.20)	3.0 (6.0)
C220ARA	79.20	1.20 (2.40)	17.20 (34.40)	5.00	5.0 (10.0)	33.40 (66.80)	2.6 (5.2)
C230A	79.20	1.20 (2.40)	17.20 (34.40)	5.20	4.8 (9.6)	40.00 (80.00)	3.5 (7.0)
C230ARA	63.40	1.50 (3.00)	21.45 (42.90)	6.25	4.0 (8.0)	33.30 (66.60)	2.5 (5.0)

[a] The receptor binding is expressed as a percentage with respect to native oLH (column A) and oLH–SPDP/LC-SPDP (column B) which were taken as 100%. Similarly the percentage protein synthesis was calculated on the basis of radioactivity incorporated in the precipitate as described in the Experimental Procedures. IC_{50} is the dose of inhibition required for 50% inhibition of protein synthesis. Numbers in parentheses show the value when corrected for the molecular weight of the conjugate assuming 1:1 complex formation.

weight, the relative activity of the conjugate varied from 25 to 43% (Table V). The chromatographically repurified conjugates (designated as C200ARA, C210ARA, C220ARA, and C230ARA) showed relatively higher receptor recognition than C200A, C210A, C220A, and C230A (Table V). As observed earlier (*15*, *17–19*), all the conjugates stimulated progesterone production in rat granulosa cells, with a tendency to enhance steroidogenesis to the same extent as native oLH (Figure 5). Their relative potencies, when corrected for the presence of gelonin in the complex, varied from 5 to 8% (Table V).

Cell-free translation experiments were carried out on native gelonin, SPDP- or LC-SPDP-modified gelonin, and their conjugates with oLH in order to determine their ability to inhibit protein synthesis, and the quantitative data are recorded in Table V. The percentage bioactivity was determined after measuring the IC_{50}. A single amino group modification resulted in more than an 85% loss in

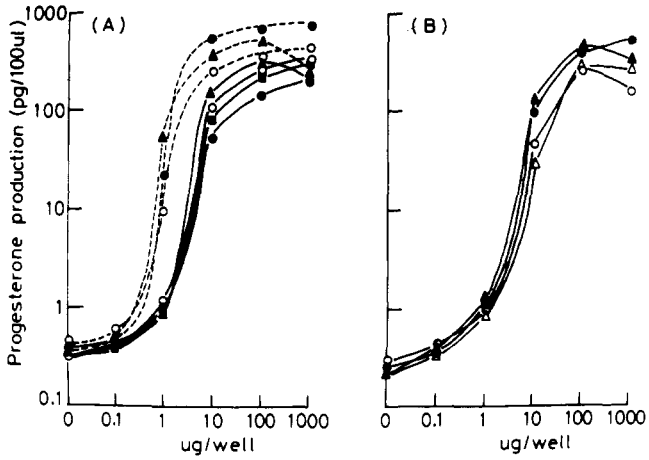

Figure 5. Steroidogenic activity of native oLH, SPDP- and LC-SPDP-modified oLH, and their conjugates with gelonin. The progesterone induction ability was determined in Leydig tumor cells as described in the Experimental Procedures: left panel, A, shows the progesterone induction ability of oLH (●- -●), oLH-SPDP (○- -○), oLH-LC-SPDP (▲- -▲), C200A (●—●), C-200ARA (○—○), C210A (▲—▲), and C220ARA (■—■); left panel, B, shows the profile of C220A (●—●), C220ARA (○—○), C230A (▲—▲), and C230ARA (△—△). Progesterone produced in 4-h incubation was quantitated by specific RIA by using specific anti-progesterone antibodies. The quantitative data are recorded in Table V.

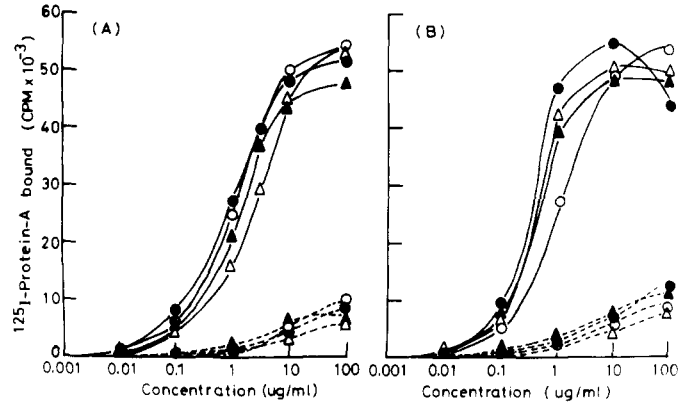

Figure 6. Saturation binding and competition ability of hormonotoxin to the Leydig tumor cells. Saturation binding, determined as described in the text, is shown by the solid line while the nonspecific binding is indicated by the dashed line. The cells were grown for 48 h and subsequently washed with serum-free medium and reacted with the indicated test materials. Following incubation and washing to remove unbound proteins, the cells were challenged with the anti-gelonin antibodies. The amount of antibody bound to the cells (via the gelonin in the conjugate) was quantitated in a subsequent reaction with ^{125}I-labeled protein-A. The number of counts (cpm) observed in each instance is a direct reflection of hormonotoxin binding via the LH receptor binding site in the conjugate and detected by the antibody directed against gelonin. The left panel shows the saturation analysis of C200A (●—●), C200ARA (○—○), C210A (△—△), and C210ARA (▲—▲). The right panel shows the analysis of C220A (●—●), C220ARA (○—○), C230A (△—△), and C230ARA (▲—▲). The radioactivity incorporated in each set is a direct reflection of hormonotoxin binding via the gonadotropin site of the conjugate as described by gelonin antibodies.

the ribosome-inactivating property (Table V). Upon conjugation with oLH, the *in vitro* protein synthesis inhibition activity was further affected (Table V). By taking gelonin–SPDP or gelonin–LC-SPDP activity as 100%, and correcting for oLH contribution in a 1:1 molar ratio in the conjugate, the activity varied from 61 to 80% (Table V).

The binding of the conjugates to the Leydig tumor cells was demonstrated with the help of gelonin antibody and by using ^{125}I-protein-A as the probe. The binding of C200A, C200ARA, C210A, and C210ARA conjugates are shown in the left panel of Figure 6 whereas, C220A, C220ARA, C230A, and C230ARA conjugates are shown in right panel of Figure 6. The nonspecific binding was determined by incubation of the appropriate quantity of gelonin (as shown by the dotted line in Figure 6).

All the conjugates saturate the receptors at a concentration of ~10 μg/mL (Figure 6). The nonspecific binding was also increased in the presence of half of the concentration of gelonin used, but this was always less than 10%. This was in agreement with our earlier observations (*17–19*). On the basis of the present observation, a concentration of 10 μg/mL of the conjugate was used for a competitive displacement analysis. The binding and displacement experiments are shown in Figure 7. Open bars indicate the binding of untreated cells and cells treated with gelonin as well as with the conjugates (Figure 7). Binding and competition experiments of C200 and C210 are shown in the lower panel of Figure 7. The upper panel of this figure shows the data for C220 and C230 hormonotoxin. The solid bars show similar binding, except the last one, which indicates the competitive ability of the native oLH with the conjugates. It is evident from the data that the presence of native oLH in the binding reaction clearly inhibited the conjugates to bind to the gonadotropin receptors of the tumor cells.

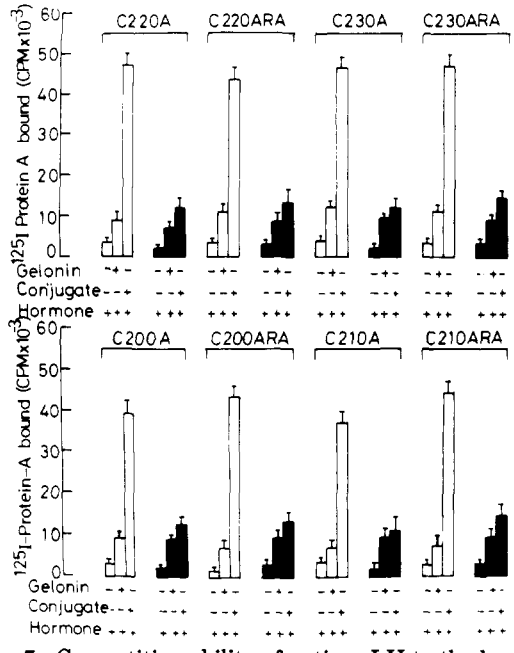

Figure 7. Competition ability of native oLH to the hormonotoxins for the binding to the Leydig tumor cells as described in the Experimental Procedures. The binding assay was performed as described in Figure 6. The binding ability of the hormonotoxin (as shown by open bar) was drastically inhibited (as shown by closed bar) in the presence of competitor (i.e., native oLH), indicating that the binding occurs via oLH.

The cytotoxicity of the hormonotoxins was evaluated using MLTC-1 cells which possess receptors for gonadotropin hormone, and their action is mediated by internalization. The cytotoxicity was determined by a leucine incorporation assay as described earlier (*15, 17–19*). All the conjugates were toxic to the cells and the IC_{50} for C200A, C200ARA, C210A, C210ARA, C220A, C220ARA, C230A, and C230ARA was 8.2×10^{-11}, 1.2×10^{-10}, 5.5×10^{-11}, 7.5×10^{-11}, 8.0×10^{-11}, 1.0×10^{-10}, 1.7×10^{-10}, and 1.3×10^{-10} M, respectively. The cytotoxicity of the

conjugates gradually decreased in the order of C200A > C220A > C210A > C230A. After rechromatography the order was C210ARA > C220ARA > C200ARA > C230ARA. In the parallel set, the conjugates incubated in the presence of native oLH did not show cytotoxicity (data not shown).

DISCUSSION

Present investigation is in continuation of our earlier efforts to design a hormonotoxin which could be selectively targeted to appropriate cells in the gonads (*15*, *17–21*). The gonadotropins such as ovine LH or hCG have been covalently cross-linked to a ribosome-inactivating proteins, by using SPDP as a cross-linking agent (*13*, *15*, *17–21*). Ovine LH/hCG was chosen as a carrier because of the selective presence of its receptor(s) in the ovary and testis (*37*, *38*) and the hormone itself can be obtained in highly purified form with extensive information available on its structure and function (*39–42*). On the basis of the structure function data that revealed that the ϵ-NH_2 groups of the α-subunit are more sensitive to chemical modification while the amino groups of β-subunit are resistant to such modifications, an attempt was made to cross-link the β-subunit to the toxin, prior to recombination with the α-subunit (*43*, *44*). But the failure of recombination with the native α-subunit forced us to use native oLH cross-linked to the toxin by using SPDP as a cross-linking agent (*15*, *17–21*). oLH and gelonin have been modified by SPDP to introduce one potential site for conjugation. Later SPDP-modified gelonin was reduced with DTT to generate SH groups which were subsequently used to obtain an oLH–S–S–gelonin conjugate. Recently, similar to SPDP, a long-chain SPDP has been introduced as a heterobifunctional cross-linking agent by Pierce Chemicals Co. Long-chain SPDP introduces a 15.6-Å-long spacer arm while SPDP could attach an arm of 8.6 Å. The hypothesis was that the introduction of a long spacer arm can effectively overcome the steric hinderance of oLH, which may help increase receptor binding and cytotoxicity. Therefore, the hormonotoxins in this study were synthesized with the help of SPDP alone (C200A and C200ARA), LC-SPDP alone (C230A and C230ARA), and both SPDP and LC-SPDP (C210, C220, C210ARA, and C220ARA) to obtain conjugates with an intermediate spacer arm, and a comparative study on immunoreactivity, receptor binding, biological activity, and cytotoxicity was carried out. As observed in case of SPDP, the primary modification of the ϵ-NH_2 group occurs in the α-subunit of oLH. But RP-HPLC analysis clearly demonstrated that the LC-SPDP-modified α-oLH was more hydrophobic in nature compared to the SPDP-modified derivative (Figure 3). The α-subunit had 11 NH_2 groups (10 ϵ-NH_2 group and 1 α-NH_2) while β-oLH possessed only 3 NH_2 groups (2 ϵ-NH_2 and 1 α-NH_2). Data reported here and in greater detail in other communications (*36*) indicated that the amino groups of α-subunit are more accessible to LC-SPDP modification than those of the β-subunit. Similar observation was also made for SPDP modification (*28*). The molar ratio of oLH and gelonin in the conjugate is equimolar, as corroborated by three different methods of assessment (Table III).

The oLH–gelonin conjugates prepared with the use of LC-SPDP were subjected to SDS–PAGE and RP-HPLC analysis. SDS–PAGE clearly revealed that the conjugate did not contain unreacted SPDP–LC-SPDP–oLH or –gelonin as a contaminant. This measurement is important at this stage since oLH, if present as contaminant, will compete with the conjugated gelonin, which eventually will reduce the receptor binding and cytotoxicity of the conjugate. Immunoreactivity, receptor binding steroidogenic activity, and cytotoxicity of oLH–gelonin conjugate prepared with the use of LC-SPDP was comparable with that of SPDP (Table V). The present investigation further demonstrated that, in case of hormonotoxin, the cytotoxicity is not affected with the use of long chain spacer arm.

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