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Specific Binding of Luteinizing Hormone to Leydig Tumor Cells*

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SUMMARY

A radioimmunoassay was used to detect luteinizing hormone (LH) bound to washed Leydig tumor cells. Tumor cell suspensions were incubated with LH at 37° and washed repeatedly by centrifugation with isotonic 0.9% NaCl solution. The tumor cells contained large quantities of LH even after they were washed sufficiently to produce a 10^6-fold dilution of unbound LH. Six washings (10^6-fold dilution) were no more effective in removing LH from the cells than three washings (10^3-fold dilution). Binding was not influenced by the temperature at which the cells were washed. The extent of LH binding was related to the number of cells, with approximately 5300 ± 960 molecules of LH bound per cell. LH binding was also proportional to the same concentrations of LH which produced a steroidogenic dose response curve. The binding constant of 1.5×10^{-8} M was considered to be higher than that expected for nontumorous tissues. Tumor cells bound more LH than did erythrocytes or thymocytes under the same conditions.

Previous studies with Leydig tumor cells indicated that the binding of luteinizing hormone to these cells was probably prerequisite for LH[1] stimulation of steroidogenesis during *in vitro* incubations (2). This conclusion was based on the observation that the stimulatory effect of LH on steroidogenesis in mouse Leydig cell tumors could be terminated only by addition of LH antiserum to the incubation medium but not by repeated washing of the cells with buffer. The time required for termination of the steroidogenic stimulus was not in itself responsible for the failure of washing to inhibit the continuing LH effects. This was shown by the fact that the stimulatory effect of adenosine cyclic 3′,5′-monophosphate on steroidogenesis was terminated immediately by the same washing procedure.

Other studies using radioiodinated gonadotropins have also indicated that they apparently bind to their "target" tissues. deKretser, Catt, and Paulson (3) have demonstrated this for Leydig cells *in vivo* with ^{125}I-LH. Eshkol and Lunenfeld (4) have observed essentially similar results for ovarian tissues *in vivo* with the use of [^{125}I]human chorionic gonadotropin.

Preliminary attempts with ^{125}I-LH failed in our hands to demonstrate adequately the specific binding of LH to the tumor cells. The availability of a radioimmunoassay procedure for LH[2] has enabled us to undertake direct measurement of physiologically active LH bound to the tumor cells.

MATERIALS AND METHODS

Preparation of Leydig Tumor Cell Suspensions—Leydig tumors, grown in C57Bl/6J mice, were minced and forced through nylon marquisette or dacron ninon as described previously (5). The larger clumps of cells were removed from the tissue by centrifugation at 160 × *g* in the cold for 5 min. The supernatant, containing small clumps of fewer than 10 to 20 cells, single tumor cells, and all the erythrocytes was centrifuged for 10 min at 320 × *g*. Most of the tumor cells and a few of the erythrocytes sedimented in the pellet. The pellet was washed twice by resuspending it in 25 ml of 0.9% NaCl solution followed by centrifugation at 320 × *g*. The cell number was determined by counting an aliquot of suspension in a hemocytometer.

Procedure for Incubation—The cells were incubated in 12-ml centrifuge tubes at 37° in the presence of varying amounts of LH in 5 ml of Krebs-Ringer bicarbonate buffer (pH 7.4). After 15 min of incubation, 5 ml of 0.9% NaCl solution were added, and the suspension was mixed and centrifuged in the cold (unless otherwise noted) for 5 min at 500 × *g*. The pellet was resuspended in 0.9% NaCl solution and the centrifugation-suspension process repeated five times to produce more than 10^6-fold dilution of any unbound LH.

Portions of the tumors not used for the binding studies were incubated at 37° in the Krebs-Ringer buffer for 1 hour in the presence or absence of 5 to 10 μg of LH per ml to test the LH responsiveness of the individual tumor being studied. Steroid synthesis was measured qualitatively by extracting the incubation medium containing the tissue with chloroform-methanol (2:1), separating this into two phases with 0.9% NaCl solution, drying the chloroform phase under nitrogen, and subjecting the lipid extract to thin layer chromatography on Silica Gel G (containing a fluorescent indicator) with hexane-ether-acetic acid

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[1] The abbreviation used is: LH, luteinizing hormone.

[2] N. R. Moudgal and A. H. Wyman, manuscript in preparation.

(60:40:3). Any response to LH was immediately obvious in terms of the relative dark spots (corresponding to 4-en-3-one steroid formation) when the plates were viewed under ultraviolet light. All the tumors discussed in this report responded to LH.

TABLE I

Response of suspended cells to LH

A cell suspension was prepared and incubated with LH in Krebs-Ringer buffer as described under "Materials and Methods." Measurement of steroids was as described previously (5). Values are the means of duplicates ± standard deviations.

Hormone	Testosterone per flask
	μg
None	0.010 ± 0.003
LH (5 μg per ml)	0.104 ± 0.015

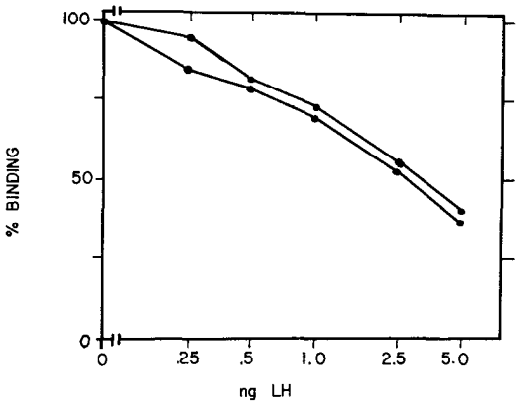

FIG. 1. Typical LH standard curves obtained using the LH radioimmunoassay. The LH assay was performed as described under "Materials and Methods" with known amounts of NIH-LH-S-16 as the standard. Each *point* represents the average of duplicate determinations.

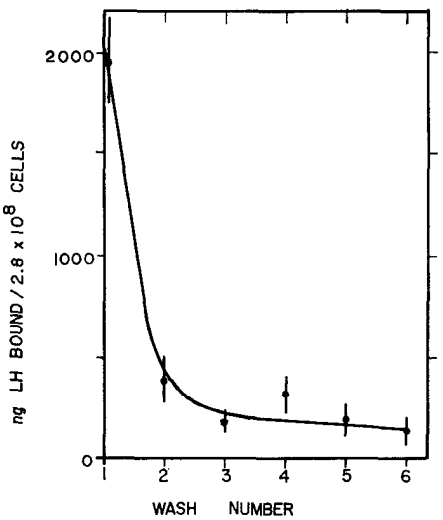

FIG. 2. Removal of LH from the tumor cells by washing. A cell suspension containing 2.8 × 10^8 cells was incubated and washed as described under "Materials and Methods." A fraction of the cells was removed at each washing to determine the effectiveness of washing at 0°. LH was quantitated by radioimmunoassay as described under "Materials and Methods." Values represent the means from three determinations at each washing. The *vertical bars* extend to the limits of the standard deviation.

In one experiment, a cell suspension prepared exactly in the manner discussed above was assayed for its ability to synthesize steroids in response to LH. In this case, material recovered from thin layer chromatography was analyzed by gas liquid chromatography as described before (5).

Procedure for Homogenization—Cells incubated with LH and subjected to the washing procedure were homogenized in cold 0.02 M glycylglycine buffer (pH 7.2) containing 0.01 M $MgCl_2$, as described by Marsh (6). The homogenate was centrifuged for 10 min at 600 × *g*. The supernatant was then centrifuged at 17,000 × *g* for 20 min, and the pellet and supernatant from this treatment were frozen and saved for LH assay. The 600 × *g* pellet was rehomogenized, rapidly frozen and thawed, homogenized again, and centrifuged at 200 × *g* for 10 min. The supernatant and pellet from this treatment were frozen for subsequent LH analysis.

Procedure for LH Analysis—The washed pellets were homogenized in 0.05 M phosphate-0.05 M EDTA buffer (pH 7.6) containing 0.2% gelatin. The LH radioimmunoassay was performed according to the method of Moudgal and Wyman,[2] which consisted essentially of incubating at 37° various amounts of cold standard LH or unknown samples with 0.1 ml of 1:7,500 or 1:10,000 dilution of a well characterized LH antiserum. Preparation and characterization of the latter have been described (7). After 12 to 24 hours incubation, 15 to 20,000 cpm of ^{125}I-labeled sheep LH (NIH-S-16), prepared according to the method of Greenwood, Hunter, and Glover (8), were mixed into all tubes, and incubation continued at 37° for 12 hours. The soluble antibody-antigen complex formed was precipitated by addition of 50 μl of 1:10-diluted normal rabbit serum and 50 μl of a goat antiserum to rabbit γ-globulin (obtained from Antibodies, Inc., Davis, California) and incubation for a further 12-hour period. All tubes were centrifuged, the supernatant aspirated, and the radioactivity in the precipitate counted in a γ counter (Packard Instrument Company). Adequate controls were included to determine the radioactivity precipitated by the second antigen-antibody system in the absence of antiserum. By means of this correction, the specific binding of ^{125}I-LH by LH antiserum was determined and compared with the values obtained for the LH standard. Tumor cells not treated with LH served as controls, together with erythrocytes and thymocytes.

RESULTS

Cell Suspensions—Examination of the cell suspensions revealed that they consisted of small clumps of tumor cells in addition to the single cells. The majority of the clumps contained fewer than 20 cells each. Since the suspensions were made without the use of enzymes, less cellular damage was expected. Cells prepared in this manner responded to LH, as can be seen from the results of a typical experiment presented in Table I.

LH Assay—Typical standard curves for the assay of LH are shown in Fig. 1. The assay reliably measured LH amounts in the range of 0.25 to 5 ng. The assay procedure had an accuracy of better than ± 20% at the extremes and ± 10% in the central range.[2] When LH was being measured in tumor fractions, a range of tissue sample was taken which provided binding curves that were parallel to the standard curve.

Removal of LH from Tumor Cells—The effectiveness of the washing procedure was tested by removing aliquots of tumor cells after each wash. As is evident from Fig. 2, washing was unable to remove all the LH from the tumor cells. After the first

two washings, the amount of LH in the tissue remained relatively unchanged. A comparison of washing at 37° with washing at 0° was included as a further check on the effectiveness of the washing procedure. As can be seen in Table II, washing at 37° did not reduce the total amount of LH bound to the tumor cells after the standard five washings. Thus, five washings at 0° were considered effective in removing all the unbound LH from the cells.

TABLE II

Effect of temperature of washing of cells on dissociation of LH from cells

Cell suspensions were prepared and incubated at 37° with 5 μg of LH for 15 min (see under "Materials and Methods"). Washing was performed as described under "Materials and Methods" with the exception that one set of cells was washed at 37° and the other at 0°.

Wash temperature	LH per 5×10^7 cells
	ng
0°	18 ± 3.0[a]
37°	28 ± 4.1

[a] Values are means ± standard deviations of triplicate determinations. One experiment is described.

TABLE III

Comparison of LH binding to tumor cells, erythrocytes, and thymocytes

The cell suspensions were incubated and washed five times (see under "Materials and Methods").

Cell type	Experiments	LH per 10^8 cells
		ng
Tumor	2 (6)[a]	30.4 ± 7.1[b]
Erythrocytes	2 (2)	1.3 ± 0.3
Thymocytes	1 (3)	Not detectable

[a] Numbers in parentheses refer to the number of determinations within each experiment.

[b] Values are means ± standard deviations for all determinations.

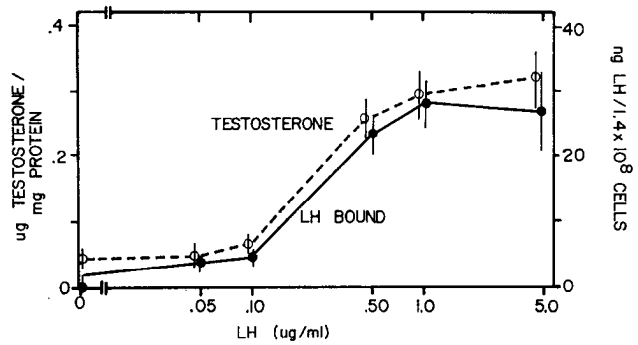

FIG. 3. Dose response nature of the LH binding. Cells were incubated with LH at concentrations of 0, 0.05, 0.1, 0.5, 1.0, and 5.0 μg per ml in a total volume of 5 ml. Washing was as described in the text. The testosterone data are those published previously (4) and are included for comparison with the LH binding data. Data for the testosterone production refer to the average of triplicate determinations at each LH concentration from one experiment. Data for LH binding refer to the average of triplicate determinations in each of two experiments. In all cases the *vertical bars* extend to the limits of the standard deviation.

Specificity of LH Binding—The specificity of the LH binding was tested by comparing the amounts of LH bound by erythrocytes and thymocytes with those bound by the tumor cells. These results are seen in Table III. Clearly, the tumor cells bind more LH than either the erythrocytes or thymocytes. This specificity was investigated further by determining the binding of varying amounts of LH to a constant number of tumor cells. Levels of LH were chosen that covered the dose response range to LH as measured by steroidogenesis. Fig. 3 shows the results of these measurements. The similarity in the shape of the binding curve with that representing the steroidogenic dose response curve is striking. Had the binding of LH been nonspecific, these

TABLE IV

Subcellular localization of LH

Cells were incubated with LH (5 μg per ml) at 37° for 15 min, washed 5 times at 0°, and fractionated as described under "Materials and Methods." The 600 × *g* pellet, almost free of whole cells, was frozen, thawed, and homogenized before fractions were isolated at 200 × *g*.

Fraction	Total LH
	ng
600 × *g* pellet	
200 × *g* pellet	33 ± 4.0[a]
200 × *g* supernatant	68 ± 4.1
17,000 × *g* pellet	3.5 ± 0.9
17,000 × *g* supernatant	Not detectable[b]

[a] Values are means ± standard deviations of three determinations per fraction in one experiment.

[b] Minimum detectable amount, 2.5 ng, due to the large volume of fraction.

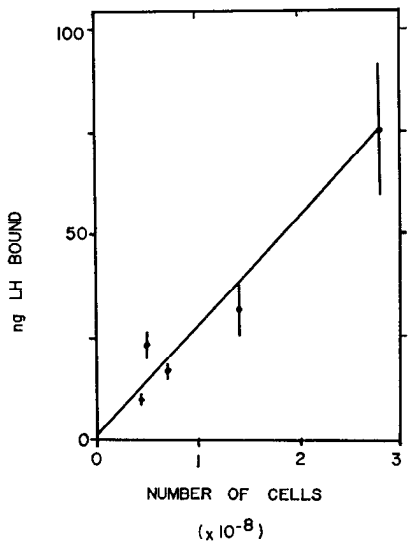

FIG. 4. Binding of LH as a function of cell number. Varying numbers of cells were incubated with 5 μg of LH and washed according to the standard procedure. A standard curve $Y = A + BX$ was calculated from the data by the method of Mood and Graybill (9). $A = 0.54 \pm 4.46$ ng of LH. $B = 2.54 \times 10^{-7} \pm 0.458 \times 10^{-7}$ ng of LH per cell. Y represents the nanograms of LH bound and X represents the number of cells. Data from several experiments have been included in this figure. Each *point* represents the mean of at least three determinations in each of two or more experiments. The *vertical bars* extend to the values of the standard deviation.

similarities might not have been observed. The binding constant was calculated to be $1.58 \pm 0.32 \times 10^{-8}$ M.

Relationship of Bound LH to Number of Cells—Experiments were designed to determine if the binding of LH was proportional to the number of cells in the incubation. Results of these experiments are seen in Fig. 4. A direct relationship is apparent between the number of cells present and the amount of LH bound. Approximately 5300 ± 960 molecules of LH were found to be bound per cell based on the slope of the line in Fig. 4. The value of the intercept is 0.54 ± 4.46 ng of LH, indicating that the line does indeed pass through the origin as would be expected if the measurements were valid.

Subcellular Distribution of Bound LH—The data shown in Table IV depict the amount of LH found in cell particulates after homogenization and differential centrifugation. Most of the LH appeared in the 600 × *g* pellet. Freezing, thawing, and homogenizing the 600 × *g* pellet caused a large amount of LH to appear in the 200 × *g* supernatant fraction. This supernatant contained small vesicles when examined with a phase contrast microscope. Nonetheless, the fact that a substantial fraction of the LH sedimented with the 200 × *g* pellet suggests that LH was bound to nuclei or to the plasma membrane (10). Since the plasma membrane contains the adenyl cyclase of many cells (10, 11) and LH has the ability to stimulate this enzyme in homogenates of luteal cells (6), the LH found in the 200 × *g* pellet is probably bound to the plasma membranes rather than to nuclei.

DISCUSSION

It is evident from the foregoing that LH specifically binds to Leydig tumor cells and that this bound LH cannot be removed by a simple washing process. The availability of a reliable LH radioimmunoassay made possible measurement of tissue bound, physiologically active LH.[2] This circumvented the use of an earlier method which relied on the binding of radioiodinated LH to tissues (3). The use of the radioiodinated LH procedure raised doubts as to the physiological activity of the labeled hormone and, more importantly, doubts as to whether the labeled hormone bound to the same sites as did the unlabeled hormone. Since the interpretation of the results presented here depends on the validity of the radioimmunoassay used, this was tested in several ways. All samples were assayed at multiple levels, and the values obtained for these aliquots were parallel to the standard dose response curve. The amount of LH found in the tumor tissue was proportional to the number of cells assayed, and the cells not incubated with LH showed no detectable LH binding. When tumor cells, erythrocytes, and thymocytes were incubated under identical conditions with LH and subjected to the same washing procedure, only the tumor cells retained substantial quantities of LH. These studies led us to conclude that we were measuring LH specifically bound to the tumor cell preparations.

The observation that the LH binding curve and the steroidogenic dose response curve bear a striking parallelism was of great interest (Fig. 4), considering that only small amounts of the added LH at any concentration were retained by the cells. Even at the lower concentrations of LH, enough total hormone was present to more than account for the amount of LH retained by the cells at the highest concentrations. Thus, it appears that a critical concentration of LH has to be reached before LH binds in sufficient amounts to cause a measurable steroidogenic response. The calculated binding constant of LH ($K_b = 1.58 \pm 0.32 \times 10^{-8}$ M) appears to be high. This is probably due to changes occurring after serial transplantation of the tumor. After eight transplants, the minimum concentration of LH required for maximal stimulation had increased from 0.05 μg per ml to 0.5 μg per ml. This change may reflect an increase in K_b. In other systems, as little as 0.01 μg per ml of LH stimulates steroidogenesis at a maximal rate (12). This suggests that K_b in nontumorous LH-responsive tissues may be as small as 10^{-10} M or less.

Our earlier observation that the steroidogenic response caused by adenosine cyclic 3′,5′-monophosphate, but not that promoted by LH (2), can be terminated rapidly by a simple washing procedure and the present observation that LH seems to bind largely to the 600 × *g* cell pellet suggest that LH is being retained by the cell membrane fraction after the washing procedure. The fact that the adenyl cyclase is probably located in this fraction (10) strengthens the idea that LH must remain attached to this membrane to stimulate adenosine cyclic 3′,5′-monophosphate, and consequently, steroid synthesis.

An attempt has been made to calculate the number of LH molecules bound per cell. Nonetheless, when compared with the number of adrenocorticotrophic hormone molecules apparently bound to adrenal cells (12), this number for LH seems excessive. This excess may be due to the fact that tumor cells might bind more LH than nontumorous ovarian or testicular cells or that the tumor cell suspensions were prepared without the use of enzymes. On the other hand, the fact that the steroidogenic dose response curve and the binding curve are similar suggests strongly that this number of LH molecules must be bound to the cells in order to obtain an LH response. Current studies are underway to assess the effects of prolactin and follicle-stimulating hormone on the binding of LH to the tumor cells. Preliminary evidence suggests that the latter is not tightly bound to the tumor cells.

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