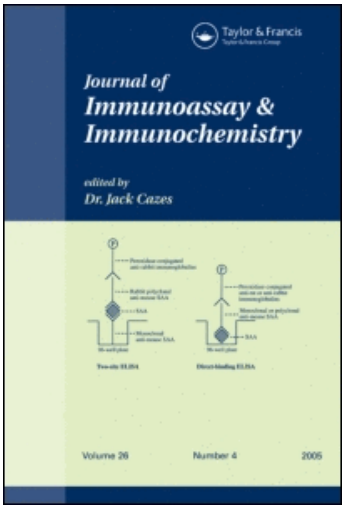

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CHARACTERISTICS OF RADIOIMMUNOASSAYS FOR THE α- AND β-SUBUNITS OF HUMAN LUTEINIZING HORMONE

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ABSTRACT

The binding characteristics and specificities of the National Hormone and Pituitary Program (NHPP) kits for the radioimmunoassay of the alpha- and beta-subunits of human luteinizing hormone (hLH-α and hLH-β) were studied, as well as the specificities of the anti-hLH and anti-human follicle stimulating hormone (anti-hFSH) antisera distributed by the same organization. The affinity constants of the anti-hLH-α and anti-hLH-β antisera were calculated at 157 ± 8.4 nM^{-1} and 109 ± 7.4 nM^{-1}, respectively. Both antisera were highly specific with regard to the other subunit. However, in the homologous hLH-α RIA, native hLH cross-reacted at 21.9%, hFSH at 17.5% and hTSH at 7.9%. The alpha-subunit of the human chorionic gonadotropin, hCG-α, was equipotent with the hLH-α standard in this assay. In the homologous hLH-β RIA, hLH showed a cross-reactivity of 14.7% while the cross-reactivities of hCG-β, hFSH and hTSH were 3.5%, 1.2% and 0.6%, respectively. The anti-hFSH antiserum was highly specific, while the anti-hLH antiserum showed non parallel competition curves. With this knowledge of the specificity of each antiserum, corrections can be properly made for the assays of hLH, hLH-α and hLH-β while the hFSH RIA can be used without correction for the presence of the three other components. (KEY WORDS: RIA, LH, FSH, alpha-subunits, beta-subunits).

INTRODUCTION

LH and FSH radioimmunoassays (RIAs) have played a pivotal role and are still essential tools in reproductive research. In order to more fully understand the mechanisms involved in the

action of LHRH and sex steroids on gonadotropin secretion, assays of biological activity are required (1) and measurements of α- and β-subunits must be at hand.

In addition to the availability of commercial reagents and kits, a large proportion of investigators use the antisera and purified hormones distributed by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK) through the National Hormone and Pituitary Program (NHPP). Since detailed data on the specificity and binding characteristics of the antibodies supplied by the NHPP for pituitary α and β-subunits are not readily available, this report describes the characteristics of the anti-human luteinizing hormone alpha subunit (anti-hLH-α) and anti-hLH beta subunit (anti-hLH-β) antisera distributed by the NHPP. It also provides some additional information on the specificities of the anti-hLH and anti-human follicle stimulating hormone (anti-hFSH) antisera available through the same agency.

MATERIALS AND METHODS

Hormones and subunits

HLH-α (batch #1), hLH-β (NIAMDD-hbeta-LH-3, AFP-2444 B), hLH (hLH-I-2, AFP 6332 B), hFSH (NIAMDD-hFSH-2) and the human pituitary gland extract, LER-907, were obtained through the courtesy of Dr. Salvatore Raiti, director of the National Hormone and Pituitary Program, NIADDK, NIH, Bethesda, Md. Highly purified alpha and beta subunits of the human chorionic gonadotropin (hCG-α and hCG-

β) as well as human thyroid stimulating hormone (hTSH) were obtained from Bio-Endo Inc., Montreal, Canada.

Antisera

Anti-hLH-α (batch #1), anti-hLH-β (batch #1) and anti-hFSH (batch #6) antisera were prepared by Dr. A.F. Parlow, Harbor-UCLA Medical Center, Torrance, CA and obtained through the NHPP, Bethesda, MD. The anti-hLH (batch #2) antiserum was prepared by Dr. V.S. Fang, University of Chicago, Chicago, Ill. and supplied by the same Agency.

Iodination

All hormones and subunits were iodinated under mild conditions with chloramine-T using a modification of the procedure described by Hunter and Greenwood (2). Briefly, 500 ng of chloramine-T and 1 mCi of ^{125}I (Amersham) buffered with 0.3M phosphate, pH 7.5, were added to 5 µg of hormone in a final volume of 40 µl and incubated for 4 minutes at 4°C with slow stirring during the first 30 seconds. The reaction was stopped by dilution with 500 µl of assay buffer (0.1 M phosphate, 0.15M NaCl, 0.1% NaN_3 and 0.1% bovine serum albumin, pH 7.1). The labeled hormones were separated from the free iodine by gel filtration on a Sephadex G-75 column (0.9 x 60 cm). Elution was carried out with assay buffer at room temperature.

Assay procedure

A liquid-phase, double-antibody procedure, was used for all assays. For the hLH-α and hLH-β systems, assay buffer (200 µl), standard hormones or serum samples (100 µl), labeled tracer (100 µl, about 40000 dpm) and antiserum (100 µl) were incubated for 24 hrs at room temperature. All dilutions were made with assay buffer, except for the antisera which were diluted in assay buffer plus 0.05M sodium EDTA containing 2.5% normal rabbit serum (NRS). In the hLH and hFSH systems, the conditions were identical except that the 24 h-incubation was carried out without the labeled hormones. At the end of the incubation, 100 µl (about 40000 dpm) of tracer was added and the incubation pursued for another 4 h prior to second antibody precipitation.

Separation of antibody-bound and free hormones was achieved with a combined polyethyleneglycol (PEG)-second antibody procedure (3). Goat anti-rabbit gammaglobulins (as second antibody, 100 µl of a 1:5 dilution) and 400 µl of 10% PEG-0.05% Tween-20 in water were added to each tube, which were mixed thoroughly and incubated for 15 to 30 minutes at room temperature prior to centrifugation. The tubes were then decanted, dried and counted in a LKB multi-gamma counter.

Antisera dilutions which yielded 40-50% binding of label were selected. The final dilutions for the anti-hLH-α, anti-hLH-β, anti-hLH and anti-hFSH sera were 1:10,000, 1:75,000, 1:250,000 and 1: 125,000, respectively. The same hormone preparations used for iodination served as standards in all four RIA systems.

Calculations and specificity

Radioimmunoassay data were analyzed using a program based on model II of Rodbard and Lewald (4). The specificity of antisera was defined by the 50% displacement ratio of the mass of standard to the mass of cross-reacting material. The specificities of the four antisera studied are summarized in Table 1.

RESULTS

Affinity of the anti-hLH-α and anti-hLH-β

Figures 1A and 2A show the effect of increasing concentrations of the respective labeled subunits on binding in the anti-hLH-α and anti-hLH-β systems, respectively. As illustrated in Figures 1B and 2B, the Scatchard plots (5) derived from these saturation curves yield affinity constant values of 157.1 ± 8.4 nM^{-1} and 109.3 ± 7.4 nM^{-1} for the anti-hLH-α and anti-hLH-β, respectively.

By equating the median effective doses (ED_{50} values) (in μCi) of the saturation curves shown in Figures 1A and 2A with the ED_{50} values (in μg) of the displacement curves obtained in the same assay (results not shown), specific activities of 97 and 53 μCi/μg were determined for the ^{125}I-hLH-α and ^{125}I-hLH-β tracers, respectively (6).

Specificity of the anti-hLH-α

The displacement curves obtained with various hormone preparations in the homologous hLH-α RIA are illustrated in Figure 3.

TABLE 1

Specificity of the radioimmunoassays for hLH-α, hLH-β, hLH and hFSH.

Hormone or subunit preparation	Assay			
	LH-α	LH-β	LH	FSH
hLH-α[1]	100	0.65	14.4*	0.09
hLH-β[1]	0.55	100	4.6*	DND
hLH-I-2[1]	21.9	14.7	100	DND
hFSH-I[1]	17.5	1.23	11.4*	100
LER-907	2.8	0.4	2.9	0.74
hCG-α	100	0.05	-	-
hCG-β	0.04	3.45*	-	-
hTSH	7.9	0.6	7.7	0.16

The potency of each hormone or subunit is expressed as a percentage (wt/wt at the ED_{50} value) of the material to which the assay is directed.
*non parallel inhibition
[1] preparation used for iodination
DND: did not displace

All gonadotropin hormone and subunit preparations gave inhibition curves parallel to that of the hLH-α standard. While hCG-α gave a displacement comparable to hLH-α with the anti-hLH-α, hLH and hFSH were equipotent, showing cross-reactivities of 21.9% and 17.5%, respectively. The other pituitary glycoprotein, hTSH, exhibited a cross-reactivity of 7.9% in this assay (Table 1). The impure human pituitary gland extract, LER-907, competed weakly with the tracer

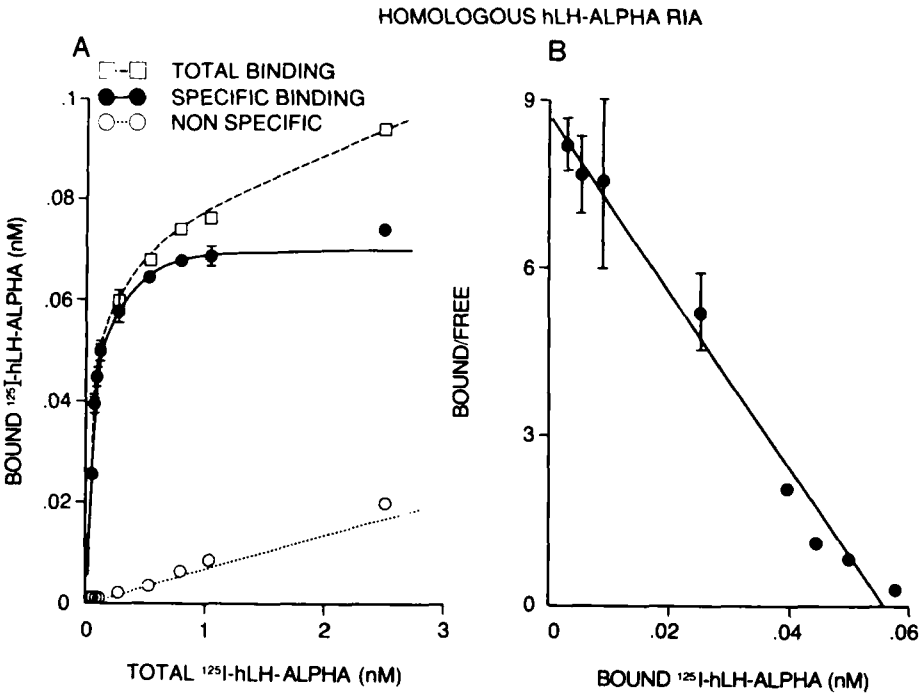

Figure 1: Panel A: Effect of increasing concentrations of ^{125}I-hLH-α on binding to a fixed concentration (final dilution 1:10,000) of anti-hLH-α antiserum. Incubations were carried out in a final volume of 0.5 ml for 24 h at room temperature prior to addition of the second antibody with 10% PEG. Panel B: Scatchard plot of the data of the saturation curve.

in this system with a low cross-reactivity of 2.8% relative to hLH-α, while hLH-β and hCG-β had a very low cross-reactivity at 0.55% and 0.04%, respectively. Dilutions of human serum gave a parallel displacement in the assay (not shown) while hLH-α was recovered quantitatively from serum (98.3 ± 3.5%, data not shown).

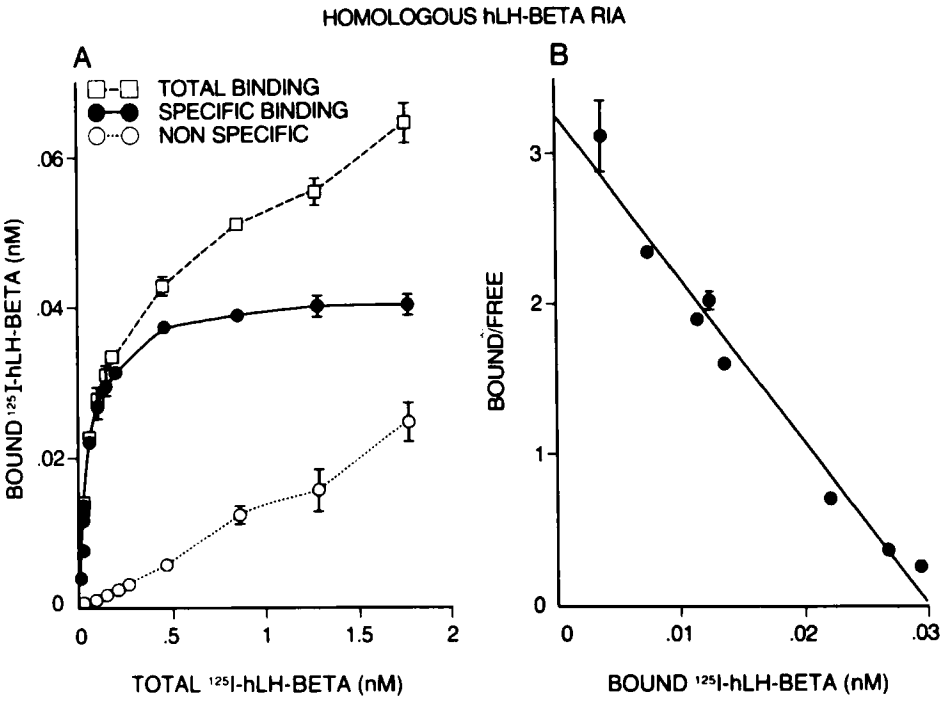

Figure 2: Panel A: Effect of increasing concentrations of ^{125}I-hLH-β to a fixed concentration (final dilution 1: 75,000) of anti-hLH-β antiserum. Incubations were carried out in a final volume of 0.5 ml for 24h at room temperature prior to separation of the antibody-bound and free labeled subunit by second antibody-PEG precipitation. Panel B: Scatchard analysis of the data of the saturation curve.

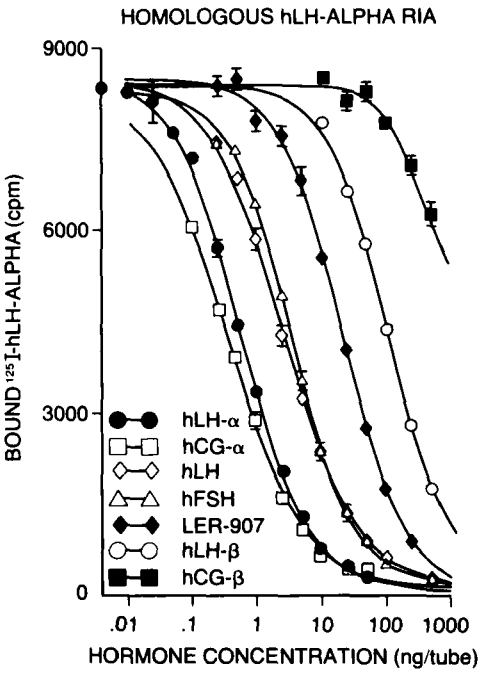

Figure 3: Cross-reactivity of a series of gonadotropin and subunit preparations in the homologous hLH-α immunoassay. Incubations were carried out as described in Materials and Methods. Results are means of duplicate determinations.

Specificity of the anti-hLH-β

The dose-response curves obtained with various hormone and subunit preparations in the homologous hLH-β immunoassay are illustrated in Figure 4. All hormones and subunits, with the exception of hCG-β, yielded a displacement parallel to that of the hLH-β standard. Native hLH cross-reacted significantly (14.7%) in this assay, while hFSH (1.2%), hLH-α (0.7%), hTSH (0.6%), LER-907

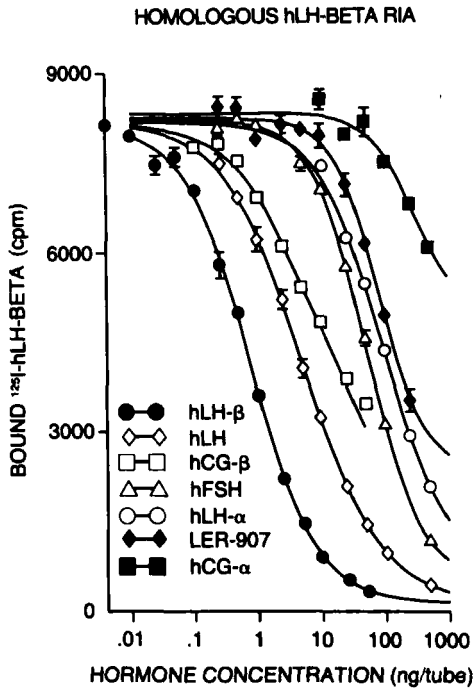

Figure 4: Cross-reactivity of a series of standard hormones and subunit preparations in the homologous hLH-β assay system. Assays were run in duplicate as described under Materials and Methods.

(0.4%) and hCG-α (0.05%) exhibited low cross-reactivities. The β-subunit of hCG (hCG-β) was considerably less potent than hLH-β, its cross-reactivity being only 3.5%.

Specificities of the anti-hLH and anti-hFSH

The above-mentioned data show that both native gonadotropins (LH and FSH) cross-react significantly in the subunit RIAs. It was then essential to determine the specificity of the anti-hLH and

anti-hFSH sera (we currently use the anti-hLH (batch #2) and the anti-hFSH (batch #6) distributed by the NHPP) in order to be able to make the appropriate corrections for LH and FSH when measuring free subunit concentrations in human serum. Figure 5 shows that the anti-hFSH is highly specific: native hLH and hLH-β do not compete with the tracer in this system, while hLH-α has a very low displacement at 0.09%. The impure human pituitary extract LER-907, which is known to contain FSH (7), yielded a displacement curve parallel to the purified hFSH standard but was 133 times less potent on a weight basis (Fig. 5).

The anti-hLH serum, on the other hand, shows a much less stringent specificity (Fig. 6). Each material used showed a significant cross-reactivity (calculated at the ED_{50} values); hFSH: 11.4%; hLH-α: 14.4% and hLH-β: 4.6%. Not shown on Figure 6 is the displacement curve generated when hTSH is used to displace the ^{125}I-hLH tracer; hTSH yielded a response parallel to the hLH standard and a cross-reactivity of 7.7% (Table 1). As expected, the pituitary extract LER-907 gave an inhibition line parallel to that of the purified hLH standard (Fig. 6), but with a 30-fold reduced potency on a weight basis. Moreover, the inhibition lines generated by the LOGIT transformation of the dose-response curves for hFSH, hLH-α and hLH-β are not parallel to that obtained with the purified hLH standard (results not shown).

DISCUSSION

The present study reports the binding characteristics and specificities of the RIA kits for the alpha and beta-subunits of

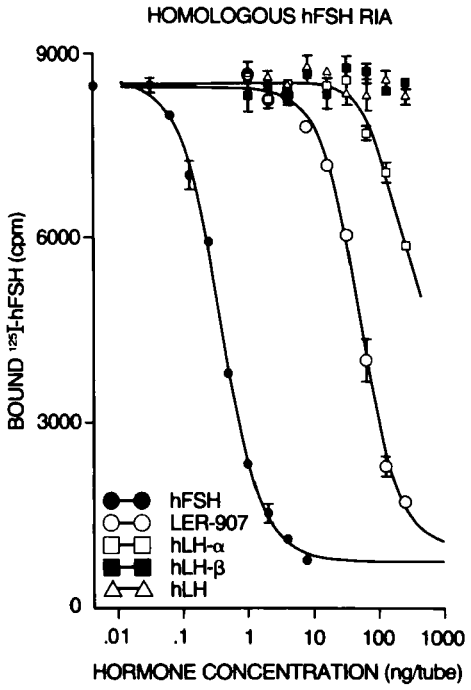

Figure 5: Specificity of the anti-hFSH (batch #6) antiserum. Incubations were carried out as described in the Materials and Methods section with increasing amounts of hFSH, LER-907, hLH-α, hLH-β or native hLH. Results are means ± SEM of duplicate determinations.

human luteinizing hormone available through the National Hormone and Pituitary Program (NHPP) as well as the specificities of the anti-hLH and anti-hFSH sera distributed by the same agency. Although other immunoassays for hLH-α (8-12) and hLH-β (7, 11, 12) have previously been characterized, no report on the RIA kits for these subunits distributed by the NHPP has, to our knowledge, been published.

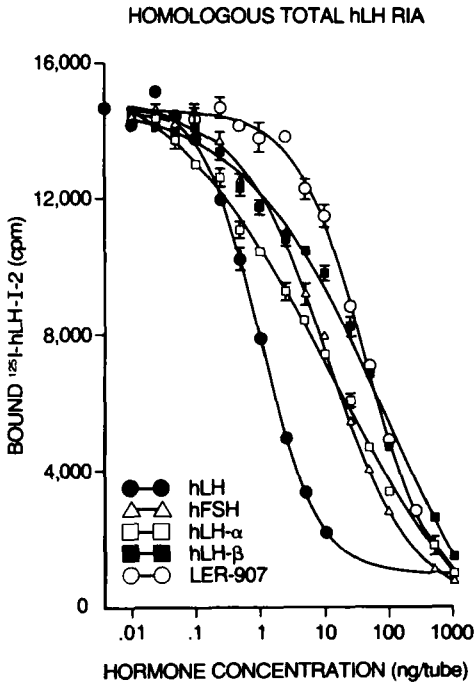

Figure 6: Dose-response curves of hLH, hFSH, hLH-α, hLH-β and LER-907 in the RIA for native hLH using the anti-hLH (batch #2) antiserum. Assays were run in duplicate as described under Materials and Methods.

Both the anti-hLH-α and anti-hLH-β sera show high affinity for their respective labeled subunit tracers. Measurements of both subunits in human serum should be accurate since standards are recovered with maximal efficiency from serum and serum dilutions give a parallel response in both assays. Of potential concern, however, is the degree of cross-reactivity found in both assay systems with reference to the native hormone hLH, and the

cross-reactivity of hFSH and hTSH in the hLH-α RIA. The observed cross-reactivities could have been due to contamination of the hormone preparations by free subunits. However, since we used highly purified (iodination-grade) preparations of native hLH, hFSH and hTSH, it is likely that the cross-reactivity exhibited by these preparations in each of the homologous subunit immunoassay systems is due to the recognition by the antisera of the α-subunit component of the native hormones. It is of interest that the cross- reactivity of the three native hormones in the hLH-α RIA is of comparable level at 21.9, 17.5 and 7.9% for hLH, hFSH and hTSH, respectively.

Since serum levels of free α- and β-subunits often rise concomitantly with the serum concentrations of the native hormones (9, 13-17), the cross-reactivity quantified above must be taken into account when expressing the results of free subunits measurements using these reagents. When using materials distributed by the NHPP, measurements of hFSH levels are accurate without any correction since the anti-hFSH (batch #6) is highly specific (Fig. 5). However, the anti-hLH serum (batch #2) cross-reacts with both the α-subunit, hFSH and hTSH. Even though the concentration of the free α-subunit can be estimated from Figs. 3-6, the use of a more specific anti-hLH would be more convenient.

The relative lack of specificity of the anti-hLH (batch #2) is not such that it precludes its use in clinical medicine where an absolute quantification of circulating LH levels is not required. In such investigations, the rather impure human pituitary

gland extract LER-907 is often used as standard (7). Moreover, recent reports (18-20) showing discrepancies between LH immunoactivity and biological potency stress the fact that LH concentrations determined by radioimmunoassay should, in many circumstances, be correlated with bioactivity using a suitable bioassay (1, 21-23).

Of the four antisera studied, the NHPP anti-hLH (batch #2) is also the only one which exhibited non parallelism in the dose-response curves generated by the free subunits and by hFSH (Fig. 6). This lack of parallelism may be explained by the fact that the subunits undergo conformational changes when combining to form the native hormone (24-28), thus possibly alterning the antigenic site (29).

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