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Establishment of an In Vitro Bioassay and Radio Receptor Assay for LH/CG in Human Sera Using Immortalized Granulosa Cells Transfected with LH/CG Receptor

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Levels of gonadotropic hormones in human sera or urine are routinely measured by radioimmunoassay or by enzyme-linked immunoassay (ELISA), which determine the immunoactivity of the hormone, but not its biological activity. We have utilized immortalized stable steroidogenic granulosa cells, which express 5–10 times more of the luteinizing hormone/chorionic gonadotropin (LH/CG) receptors than the primary cells, to develop a biological assay and radioreceptor assay for this hormone. We found that stimulation of cells expressing LH/CG receptor with increasing doses of human LH or human CG resulted in a dose-dependent increase of cAMP and progesterone with an ED_{50} of 30 and 57 mIU/mL, respectively. These dose–response data served as calibration curves for measuring the gonadotropin bioactivity in human serum samples at concentrations as low as 1–5 mIU/mL. We found a close correlation between LH levels measured by enzyme immunoassay (EIA) and the in vitro bioassay in normal cycling and menopausal women, as well as in normal adult men. Also, a close correlation was found between the EIA and the in vitro biological assay of hCG in pregnant women. In addition, we have developed a radioreceptor assay (RRA) for this hormone using enriched cell membranes of the appropriate cell line, which corresponds well to both the EIA and the bioassay in human sera. Deglycosylated hCG was fully active in RRA, but failed to activate cAMP response in these cells, demonstrating the importance of the bioassay in the biologically inactive form of gonadotropins. We believe this novel in vitro bioassay of gonadotropic hormones will serve as a useful tool for a more comprehensive set of assays that will determine not only the amount, but also the possible modulation in bioactivity of the gonadotropin associated with gonadal failure and miscarriage.

Key Words: hLH/hCG bioassay; cAMP; progesterone; receptor assay; immortalized granulosa cells.

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Introduction

Luteinizing hormone (LH) is a member of the family of glycoprotein hormones that share many structural similarities with the other members of the family, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and chorionic gonadotropin (CG). Each of these proteins is composed of a common α-subunit and a β-subunit that confer the biological specificity to the hormone (Pierce and Parsons 1981). Each gonadotropin subunit possesses N-linked carbohydrate chains attached to specific asparagine residues (Green et al., 1986; Green and Baenziger, 1988). This results in large heterogeneity of carbohydrate moieties (Wilson et al., 1989). The heterogenic form of the glycoprotein hormone is present not only in the pituitary gland (Keel and Grotjean, 1984; Wide, 1985), but also in blood (Strollo et al., 1981; Wide, 1982; Suginami et al., 1985) and urine (Harling et al., 1986). The molecular isoforms exhibit differences in isoelectric properties, plasma half-life, receptor binding activity, and bioactivity (Costagliola et al., 1994).

Hormonal environment of the pituitary gonadotrophs affects not only the amount of LH released, but also the relative abundance of LH isoforms (Reader et al., 1983; Suginami et al., 1985; Dufau and Veldhuis, 1987). The pituitary in polycystic ovarian disease (PCOD) patients releases pulses of biologically inactive LH in response to GnRH stimulus (Imse et al., 1992). The presence of immunoreactive, but biologically inactive LH has led to the identification of a defective LHβ gene in an infertile patient (Weiss et al., 1992). These studies indicate the importance of analyzing the bioactive levels of gonadotropin in circulation. Immunoassays that are commonly employed (Wen-

nink et al., 1988; Apter et al., 1989) to determine serum LH levels are largely insensitive to changes in bioactive forms that may be of clinical significance. In addition, there have been reports about variation in the bioactive/immunoactive ratios in patients with abnormal gonadotropin secretion (Haavisto et al., 1990; Jaakkola et al., 1990). Although various bioassays have been reported using rodent Leydig/granulosa cells (Bajpai et al., 1974; Van Damme et al., 1974) or human granulosa-luteal cells (Ding et al., 1991), discordant values were obtained by these assays. Jia et al. (1993) have developed a luminescence-based LH/CG bioassay using human fetal kidney cell line 293 transfected with LH/CG receptor and a luciferase reporter gene containing the cAMP responsive elements (Jia et al., 1993). This assay has been used to measure the CG levels in women and nonhuman primates. However, in such an assay, the steroidogenic response to the gonadotropic hormone cannot be measured.

As an alternative to the bioassays described, we sought to develop a sensitive in vitro bioassay, using a granulosa cell line stably expressing the LH/CG receptor. This cell line (GLHR-15) was established in our laboratory by triple transfection of rat granulosa cells with SV40 DNA, Ha-*ras* oncogene, and a plasmid containing the rat LH/CG receptor gene (Suh et al., 1992). They express about 5- to 10-fold more LH/CG receptor compared to the primary granulosa cells, and fully retain their cAMP and steroidogenic response to LH/CG stimulation. These cells can therefore serve as a useful system for measuring both the potential of a gonadotropin molecule to bind to its specific receptor as well as to activate the hormone-sensitive adenylate cyclase and the induction of steroidogenesis, thus permitting a more comprehensive analysis of the gonadotropin bioactivity in a well-defined cell system. We now report the validation of the in vitro bioassays to measure human serum LH, using both cAMP and progesterone as the end point of the assay. In addition, we have also developed a radioreceptor assay (RRA) using the membrane preparation of the cell line.

Results

LH Dose–Response Curve for cAMP and Progesterone Production in GLHR-15 Cells

The LH receptor transfected cells responded to increasing concentrations of human LH (hLH) in production of both cAMP and progesterone in a dose-dependent manner (Fig. 1). Stimulation of cells with hLH resulted in a significant elevation of intracellular cAMP levels, which was already evident at 1 mIU/mL of the hormone (Fig. 1A, inset). Maximal accumulation of cAMP, 32-fold above the basal levels, was obtained at 300 mIU/mL. It should be noted that addition of gonadotropin-free serum to the assayed culture, during the hormonal stimulation period, did not significantly affect the levels of cAMP production (data not shown).

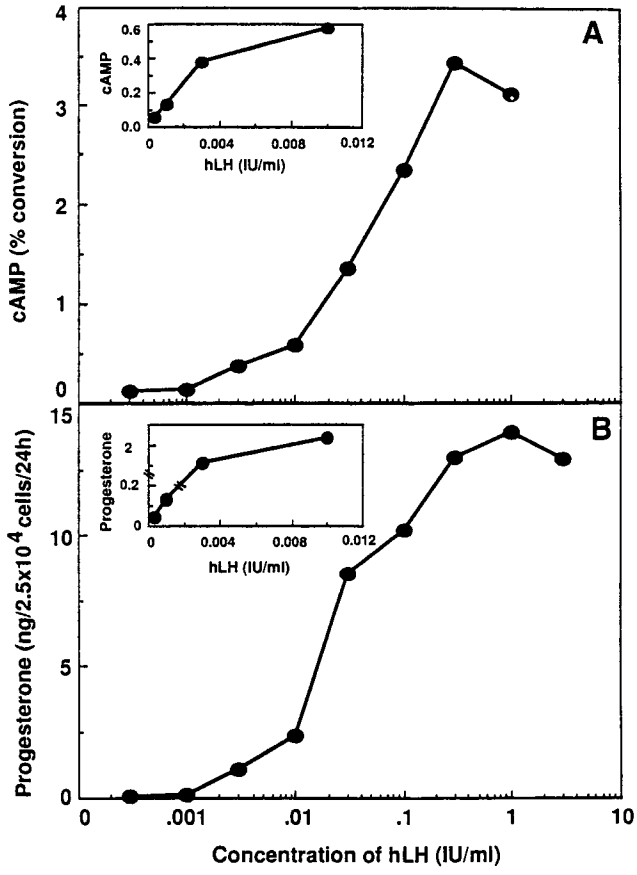

Fig. 1. A typical dose–response curve of LH stimulation by GLHR-15 cell line. **(A)** Cells were stimulated with increasing concentrations of hLH (Pergonal®, Teva Ltd., Petah Tibra, Israel) for 30 min and the conversion of ^{3}H-adenine to cAMP was measured as indicated in Materials and Methods. **(B)** Cells were cultured in presence of hLH for 24 h, and progesterone secreted in the medium was determined by RIA. The values are mean of duplicate, whereas the variation from the mean did not exceed 12%.

Stimulation of progesterone production by hLH was also evident at 1 mIU/mL concentration of the hormone (Fig. 1B inset), reaching maximum values (190-fold increase over basal) at concentrations as high as 1 IU/mL (Fig. 1B). Gonadotropin-free serum did not significantly affect the steroidogenic response of the GLHR-15 cells. The endogenous level of progesterone in the tested human sera was monitored and was subtracted from the progesterone level found in the culture media of cells incubated with the test sample. This procedure was essential in order to measure accurately the net contribution of the gonadotropin-induced progesterone production measured in the culture media of the cells. Human FSH (hFSH) had no effect on the induction of cAMP and progesterone stimulation even at a concentration as high as 1 IU/mL (Suh et al., 1992).

Effect of Receptor Concentration and Incubation Time in RRA

Addition of constant amount of ^{125}I-human CG (^{125}I-hCG) to increasing amounts of membrane-enriched fractions of

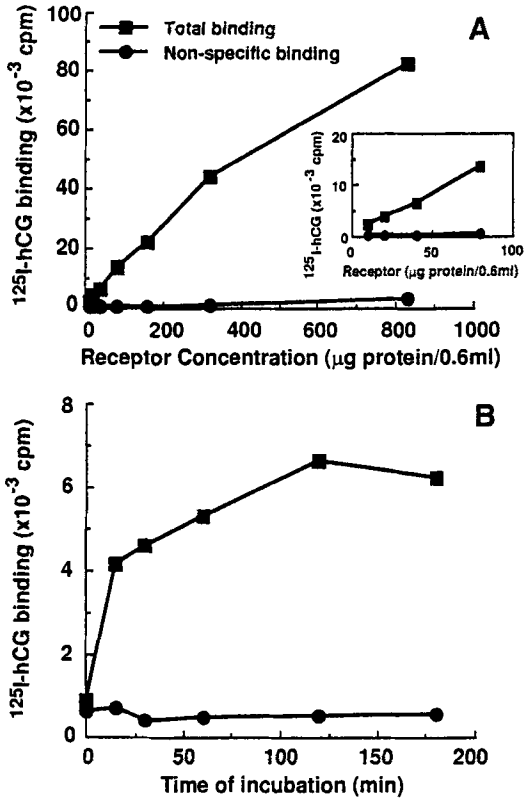

Fig. 2. Effect of membrane protein concentration and incubation time on the binding of ^{125}I-hCG to membrane-enriched fractions of immortalized granulosa cells expressing the LH/CG receptors. **(A)** 10–830 µg of the membrane were added to ^{125}I-hCG (100,000 cpm) and incubated at 37°C for 1 h followed by separation of free and membrane-bound hormone, and the amount of radioactive hCG associated with the membrane was monitored by a γ-counter. **(B)** Aliquots of membrane-enriched fraction (80–100 µg protein) containing the LH/CG receptor were preincubated for 15 min at 37°C either with buffer alone (total binding) or with 100-fold excess of unlabeled hCG (nonspecific binding) followed by the addition of fixed amount of labeled hCG and incubation continued for varying time. At the end of each time-point, the bound and free ^{125}I-hCG were separated by high-speed centrifugation, and the membrane-bound radioactive hCG was measured. The values are mean of duplicate, whereas the variation from the mean did not exceed 5%.

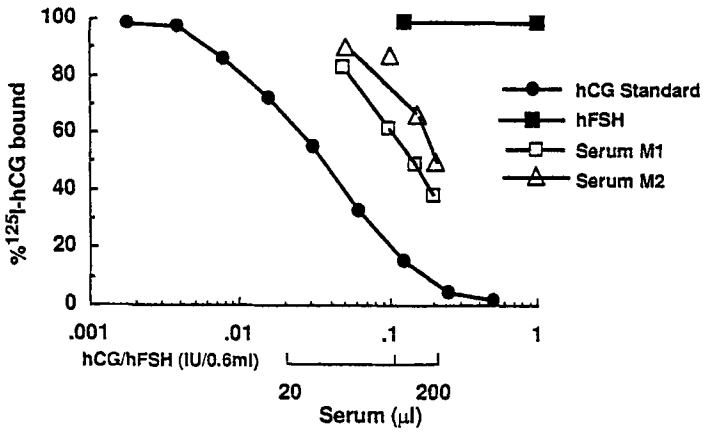

Fig. 3. Calibration curve of LH/CG receptor assay using membrane fraction of immortalized granulosa cell expressing the LH/CG receptor (GLHR-15). Varying concentrations of hCG (2–500 mIU) or serum samples (50–200 µL) were preincubated with 80 µg of the membrane for 15 min at 37°C in a volume of 0.5 mL, followed by the addition of the ^{125}I-hCG (100,000 cpm/100 µL), and incubation was continued for a further 1 h. The membrane-bound and free ^{125}I-hCG were separated as described in Materials and Methods, and the bound radioactive ^{125}I-hCG was measured in a γ-counter. hFSH (0.1–1 IU/tube) was also included in the assay to check the specificity of the assay. Data are mean of duplicate determination, whereas the deviation from the mean did not exceed 5%.

receptor led to a dose-dependent increase in the binding of ^{125}I-hCG to the membrane (Fig. 2A). As demonstrated in the inset of Fig. 2A, at 80 µg of membrane protein, 25-fold increased total binding over the nonspecific binding was observed. At a higher membrane concentration, a significant elevation in nonspecific binding was evident. Therefore, we chose this amount of membranes as a standard amount of receptor for the RRA. It should be noted that for the competitive assay, we preincubate the membranes with unlabeled hormone for 15 min before adding the labeled hormone rather than adding them together, which increased significantly the sensitivity of the assay.

The binding of the radiolabeled hormone to the membrane receptor was time-dependent, reaching 80% of maximal binding during 1 h of incubation of the membrane fraction with the radioactive hormone at 37°C, whereas the nonspecific binding remained low and constant during this period. Thus, the optimal condition for the RRA used for human sera was 15 min preincubation at 37°C of the standard or the serum samples with 80 µg membrane protein, followed by an additional hour of incubation in the presence of the labeled hormone with shaking of the sample in a water bath. The assay was terminated by the addition of 1 mL ice-cold buffer followed by separation of free and membrane-bound hormone by centrifugation.

Specificity and the Ability of the Receptor Assay to Measure LH Levels in Human Serum

A representative standard curve of hCG displacement of labeled hCG is shown in Fig. 3. The minimal detectable dose of hCG was 4.5 mIU and the ED_{50} was 39 ± 2.4 mIU. FSH, even at a high dose of 1 IU/tube, did not show any displacement of the ^{125}I-hCG binding to membrane, indicating the assay to be specific for LH/CG. Addition of 200 µL of gonadotropin-free serum reduced slightly the total binding when compared with the standard medium of incubation containing 1 mg/mL BSA. Reduction in the binding to the same extent was observed when the gonadotropin-free serum was added to the different standard test tubes containing increasing amount of unlabeled hormone. Hence, the data were identical when they were plotted as percent ^{125}I-hCG bound either in the absence or the presence of 200 µL gonadotropin-free serum (data not shown). In any case, when human sera were assayed, 200 µL gona-

dotropin-free serum were added to the control assay, and the extent of displacement was calculated from the specific binding obtained in the presence of gonadotropin-free serum.

Addition of 50–200 μL of serum from two postmenopausal women yielded a displacement curve that was parallel to the standard displacement curve (Fig. 3). When the amount of LH was calculated using the different amounts of the sera utilized in the assay (50, 100, 150, 200 μL) for the two menopausal women, it was found to be 245 ± 12 and 131 ± 21 mIU/mL. This would indicate that RRA for LH measurement can be performed at a range of 50–200 μL serum.

Effect of hCG and Deglycosylated hCG (dghCG) in the Bioassay

Since the bioactivity of the gonadotropin depends to a large extent on the sugar content of the molecule (Sairam, 1989) and since the naturally occurring forms can markedly vary in their sugar content (Courte and Willemot, 1972; Dufau and Veldhuis, 1987; Costagliola et al., 1994), we compared the effect of chemically deglycosylated hCG both in the RRA and in the bioassay. hCG and dghCG were able to displace the binding of labeled hCG to the membrane receptor in a dose-dependent manner (Fig. 4A). The concentrations required for 50% displacement of ^{125}I-hCG were 1.14 and 0.94 ng/tube for hCG and dghCG, respectively, which indicated that deglycosylation of the hormone had no effect on the receptor binding ability. However, when cAMP responses elicited by hCG and dghCG were compared, it was found that no significant response was obtained with dghCG at a concentration of 0.8 ng/mL, although the response to the intact hormone was 12 fold over the basal. At concentration of 50 ng/mL, the cAMP response to the native hormone was 190-fold above basal, but the deglycosylated reached only to 17-fold over the basal.

The ability of the cells to secrete progesterone in response to increasing concentration of dghCG was also blunted when compared to the effect of hCG, although the reduction was less pronounced at high concentrations of the hormone. Although 0.39 ng/mL of the intact hCG was able to increase progesterone by 62 pg/mL (over basal), the increase by dghCG stimulation amounted only to 3 pg/mL (20-fold increase in hCG vs dghCG). At 13 ng/mL, hCG-induced progesterone production was 127 pg/mL, but the dghCG elevated progesterone production by 74 pg/mL.

Measurement of Human Serum LH

Table 1 provides the cumulative data of LH levels obtained by EIA and the different assays described in this article. Normal male and female had low LH levels and the postmenopausal female serum had an elevated concentration of LH. As expected, the pregnant female had the maximal level of gonadotropin owing to the presence of circulating CG. When serum samples of pregnant

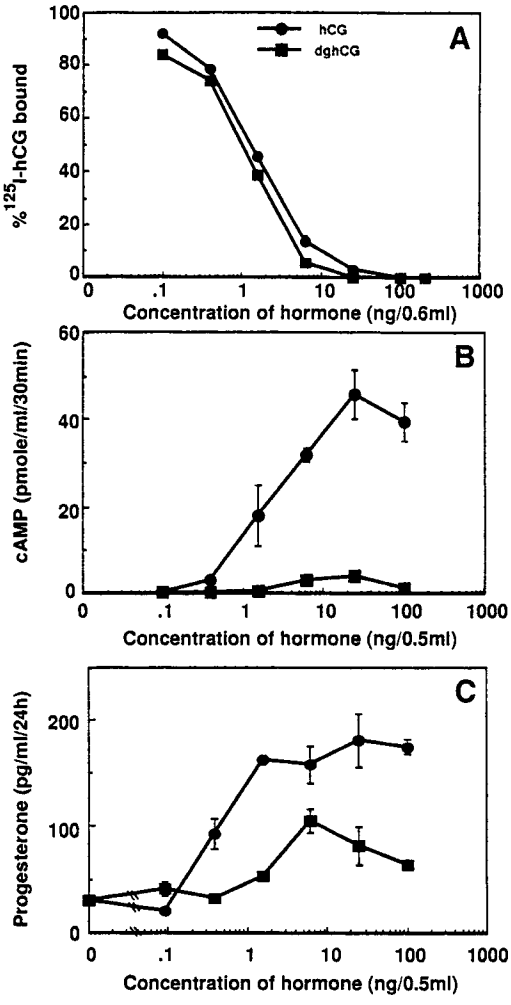

Fig. 4. Comparison of hCG and deglycosylated hCG (dghCG) in bioassay and RRA using immortalized granulosa cell (GLHR-15) expressing the LH/CG receptor. **(A)** Displacement of ^{125}I-hCG binding to the receptor-enriched fraction by hCG/dghCG. (**B** and **C**) Dose-dependent stimulation of intracellular cAMP level (B) and progesterone secretion (C) by the cells in response to hCG/dghCG. Each point is mean of duplicate in panel A, and the deviation from mean was <5%. The data in B and C are mean ± SEM of triplicate determination.

women were tested, dilutions of 1:20, 1:50, and 1:100 were used. Essentially similar values for the gonadotropin bioactivity were obtained when different dilutions of the sera were utilized in the bioassay. When the experimental values were compared between the EIA and the bioassay and the receptor assay, an appreciable degree of correlation could be observed between the four different assays performed on the human samples (Table 1). When EIA was compared to the progesterone bioassay, there was a very close correlation between the two assays. In the cAMP-based assay, the levels were slightly higher compared to the EIA, except for the male. Similarly, in the RRA, elevated levels were observed compared to all the other assays. However, the values obtained by different assays correlate with the physiological status of the individuals.

Table 1
Comparison of LH/CG Levels as Measured by EIA, RRA, and In Vitro Bioassays in Human Sera[a]

	LH/CG, mIU/mL, bioassay			
	EIA	Progesterone	cAMP	Receptor assay
Female				
Menopause	38.70 ± 4.40	32.88 ± 10.90	43.11 ± 9.59	81.11 ± 11.77[b]
n	19	9	9	17
Pregnant	3371 ± 922	2656 ± 1429	5527 ± 2434	8633 ± 3199
n	3	3	3	3
Normal	5.22 ± 0.95	3.02 ± 1.06	11.25 ± 1.25	19.37 ± 5.94[b]
n	9	4	4	4
Male				
Normal	3.10 ± 1.22	3.60 ± 0.77	1.87 ± 0.87	18.16 ± 3.6[c]
n	6	4	4	5

[a]Standard dose–response curve was performed in parallel to each set of assays using the same batch of cells/cell membrane. Sera samples were stored at –20°C until assayed. Data are mean ± SEM.
[b]$p < 0.05$.
[c]$p < 0.01$, compared to EIA, Paired Student's *t*-test. All other p values were higher than 0.05.

Table 2
Characteristics of the Bioassay and the RRA

	Bioassay		
	cAMP	Progesterone	Receptor assay
ED_{50}[a]	30 ± 6.2 mIU	57 ± 8.1 mIU	39 ± 2.4 mIU
Sensitivity	1 mIU	1 mIU	4.5 mIU
Range	1–300 mIU	1–1000 mIU	4.5–125 mIU
Interassay CV[b]	10.6%	12.3%	8.8%
Intraassay CV[b]	7.4%	8.2%	6.2%

[a]ED_{50} is the mean ± SEM of five independent standard displacements of the RRA and seven dose–response curves of the bioassay.
[b]The interassay and intraassay CV were validated using sera samples from six different individuals.

Reproducibility of the Bioassay

We found that the dose–response curves of the bioassays and the RRA were fully reproducible using different batches of cells or cell membranes derived from different batches of cells (data not shown). There was no appreciable change in ED_{50} and sensitivity of the assay with multiple passage of the cells. Regarding the steroidogenic response of the cells, there was some decrease after 20 passages of the cells. Therefore, we used a new batch of frozen cells after 20 passages. In any case, all data on human sera were calculated from calibration curves that were obtained in parallel from the same batch of cells. The calculated ED_{50}s for LH stimulated cAMP formation and progesterone production were 30 ± 6.2 and 57 ± 8.1 mIU/mL (Table 2). The sensitivity for both cAMP- and progesterone-based bioassays was 0.001 IU/mL and the range was 0.001–0.3 and 0.001–1 IU/mL, respectively. The interassay coeffecients of variation as measured using sera obtained from normal and postmenopausal women were 10.6 and 12.3% for the cAMP- and progesterone-based bioassays, respectively. The intraassay coefficients of variation were 7.4 and 8.2%, respectively. The ED_{50} and the sensitivity of the receptor assay were 39 ± 2.4 and 4.5 mIU hCG, respectively, whereas the range of the standard displacement curve was 4.5–125 mIU hCG. The interassay and the intraassay coefficients of variation as determined using human sera were 8.8 and 6.2%, respectively (Table 2).

Discussion

In this article, we demonstrate that LH/CG bioactivity can be measured both in isolated forms and in human sera using steroidogenic cell lines, expressing about 10-fold the number of LH/CG receptors compared to primary granulosa cells (Suh et al. 1992). The high sensitivity of the assay that can detect at concentrations as low as 1 mIU of the hormone/mL make it a useful assay for the detection of a

low concentration of the hormone in normal cycling women and in males. This sensitivity is similar to the EIA. The possible interference of the serum in the cAMP assay was circumvented by using the conversion of intracellular labeled ATP to cAMP (Salomon, 1991), rather than measuring directly the concentration of unlabeled cAMP by the protein binding method (Gilman, 1970). The sensitivities of the cAMP and progesterone response were found to be similar for both LH and hCG (data not shown), suggesting that the same calibration curves can be used for both hormones. FSH even at a relatively high dose failed to stimulate cAMP or progesterone production in these cells, indicating the high specificity for LH/CG in the assay (Suh et al., 1992). In addition, since the cells are homogenous, the interassay variability observed in the primary cell bioassays is reduced, thus making this assay more consistent in measuring serum LH and hCG compared to the bioassays based on primary Leydig cells or granulosa cells (Bajpai et al., 1974; Van Damme et al., 1974; Ding et al., 1991).

Application of RRA for LH and CG in macaques and other species was recently demonstrated using membrane-enriched fraction of sheep corpus luteum (Selvaraj and Moudgal, 1993). The use of membrane-enriched fraction rather than the intact cells is preferable because of the internalization of the hormone–receptor complex, which may interfere with the displacement of the labeled hormone by the unlabeled hormone (Conn et al. 1978; Amsterdam et al. 1979). In the present work, we demonstrate that using GLHR15 cell membranes as a source for the LH/CG receptor, we could develop an RRA with a sensitivity of 4.5 mIU concentration of hCG and a very high specificity to LH/CG, with no displacement by hFSH, even at high concentrations of 1 U/mL. Moreover, preparation of the membrane-enriched fraction of GLHR 15 cells is less tedious than isolating membranes from sheep corpus luteum (Selvaraj and Moudgal, 1993). Although 80 μg of membrane protein were used for the standard assay, it was evident that such an assay can be performed using a lower amount of membrane protein, such as 10 μg/assay. Incubation time of the membrane could be reduced to 1 h, since 80% of maximal binding was already achieved during this period.

The markedly reduced potency of the deglycosylated hormone to activate the hormone-sensitive adenylate cyclase is in line with the earlier observations from various laboratories, including ours (Moyle et al., 1975; Keutmann et al., 1983; Zor et al., 1984; Kalyan and Bahl, 1985; Sairam and Bhargavi, 1985). The effect of hCG and dghCG on cAMP response, progesterone production, and receptor binding provides a valid check for the bioassay system. A bioassay that can discriminate different degrees of glycosylation of the hormone may be essential for the diagnosis of possible reduced potency of the hormone in patients suffering from impotency (Fabbri et al., 1988), luteal-phase deficiency (Soules et al., 1989), and various other situations of gonadal failure. Although deglycosylation had drastically reduced the cyclase activation, low steroidogenic response was observed in the GLHR15 cells. Some disagreement between cAMP and progesterone response has been reported earlier (Sairam, 1989).

Using human sera, we demonstrate a reasonable agreement between the EIA, cAMP, and progesterone assays. The general higher values of the SEM in the cAMP and progesterone assays may arise from the different bioactivities of the hormone in different individuals. The RRA values were in most cases significantly higher than values obtained by other assays, from yet unknown reasons, and in line with RRA performed recently for the content of LH/CG in monkeys (Selvaraj and Moudgal, 1993), although the relative ratios compared to EIA were well preserved in women and to a lesser extent in male measuring the cAMP response. The discrepancy in the male may arise from less accuracy in measurements performed at extremely low concentrations of LH. However, one cannot exclude the possibility that male LH may possess a lower bioactivity than female LH in the cAMP bioassay. Variability in immunoreactive and bioactive LH content was demonstrated in breast-feeding women (Seron-Ferre et al., 1995) as well as in human urinary menopausal gonadotropin preparations (Cook et al., 1988; Rodgers et al., 1995). A discordant level of serum bioactive LH in men as measured in different in-vitro bioassay systems was also reported (Ding et al., 1991). Recently, Seron-Ferre et al. (1995) have observed diminished LH biopotency. These findings stress the necessity of a convenient bioassay system for both LH/CG and FSH, in normal individuals as well as those with gonadal failure.

In conclusion, the bioassay described in this article seems to be advantageous over the conventional bioassays using either primary granulosa or Leydig cells (Bajpai et al., 1974; Van Damme et al., 1974) in that it utilizes a more uniform population of cells. Moreover, this assay seems to be far superior to the current receptor-transfected assay (Jia et al., 1993) in that it provides a means to test receptor binding, cAMP, and progesterone response simultaneously in the same system of immortalized granulosa cells. It is hoped that the immortalized granulosa cell lines established in our laboratory, which respond either to LH/CG or to FSH (Suh et al., 1992; Keren-Tal et al., 1993), will serve as a useful tool for providing an in vitro bioassay on a more routine basis.

Materials and Methods

Materials

The culture wares were obtained from Nunclon, Denmark. The medium was provided by the biological unit of our institute. Fetal calf serum (FCS) was purchased from Biological Industries Co. (Beit Haemek, Israel). Highly purified hCG-CR-123 was kindly provided by E. Canfield

of Columbia University, NY. Pergonal was a kind gift of Teva Ltd., Israel. Chemically deglycosylated hCG and hCG were generous gifts of W. E. Merz, of the University of Heidelberg, Heidelberg, Germany (Merz, 1988; Schwarz et al., 1991). Tritiated cAMP (28 Ci/mmol) and progesterone (110 Ci/mmol) were purchased from Amersham, Buckinghamshire, UK. Antiserum for progesterone was kindly provided by F. Kohen of the Department of Bioregulation, the Weizmann Institute of Science. Phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), adenosine 5'-triphosphate (ATP), and adenosine 3':5'-cyclic monophosphate (cAMP) were obtained from Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade.

Cell Culture and Bioassay

The cell line (GLHR 15), which was developed earlier (Suh et al., 1992) by triple transfection of primary rat granulosa cells with SV40 DNA, Ha-*ras* oncogene, and an LH receptor expression plasmid, was utilized in this study. Cells were grown in Nunc Petri dishes (100 mm) containing 8 mL DMEM/F12 medium (1:1) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% FCS. For the bioassay, cells were cultured in Nunc 24-well plates (2.5×10^4 cells/well/0.5 mL) in medium containing 5% FCS for 24 h at 37°C in a CO_2 incubator with 5% CO_2 and 95% air, and the medium was replaced by serum-free medium containing 0.1 m*M* 3-isohathyl-1-methylxanthine (IBMX) and varying concentrations of hLH. Following 24 h of culture, the medium was aspirated and stored at –20°C until progesterone assay was carried out. When serum samples were included, 50 μL of the samples were used for measurement. For control, the same volume of either heat-inactivated FCS or gonadotropin-free human serum obtained from a patient diagnosed as panhypopit owing to an invasive tumor was used.

cAMP-Based Bioassay

cAMP was measured by the amount of the conversion of the intracellular labeled ATP to cAMP as described earlier (Salomon, 1991). After 24 h of culture in a 24-well Nunc plate, cells were washed and incubated in serum-free medium containing (2-^{3}H)adenine (20 μCi/mL). Following overnight stimulation, medium was replaced by serum-free medium containing 0.1 mg/mL BSA, 0.1 m*M* IBMX, and varying concentrations of hLH (0.001–1 IU/mL) or the test samples in a total volume of 0.25 mL, and incubated for 30 min. At the end of the incubation, medium was discarded and the reaction terminated by the addition of 1 mL ice-cold 2.5% perchloric acid containing 0.1 m*M* cAMP. The perchloric acid was neutralized with the appropriate amount of 1*M* Tris/4.2*M* KOH. The labeled cAMP was measured after isolation of the intracellular cAMP by sequential chromatography in Dowex-50 cation exchanger and neutral alumina. ^{3}H-cAMP was calculated as a fraction of total ^{3}H-adenine uptake/well (Zohar and Salomon, 1992; Aharoni et al., 1995).

The effect of dghCG on the intracellular cAMP level was estimated by the cAMP binding protein method as described elsewhere (Amsterdam et al., 1979, 1988; Keren-Tal et al., 1993). In brief, after 24 h of culturing in 24-well plates, the cells were replaced with serum-free medium containing varying doses of dghCG/hCG and 0.1 m*M* IBMX, and incubated for 30 min. Following incubation, the medium was aspirated and the cells scraped with a rubber policeman into 50 m*M* sodium acetate buffer (pH 4.0) containing 0.1 m*M* IBMX, boiled at 95°C for 10 min, and then centrifuged to obtain the supernatant containing the intracellular cAMP. This was estimated in the assay using cAMP binding protein and ^{3}H-cAMP, as described by Gilman (1970).

Preparation of Membrane-Enriched Fraction

The cells grown in 150-mm plates were lysed by adding 0.02*M* phosphate buffer containing 1 m*M* PMSF and 2 m*M* EDTA, pH 7.4, and the cells were scraped using a rubber policeman. The cell lysate was centrifuged at 30,000*g* for 20 min, and the pellet was suspended in PBS with 1 m*M* PMSF and recentrifuged. The final membrane pellet was suspended in PBS containing 1 m*M* PMSF and stored at –70°C until further use.

Iodination Procedure

Purified hCG was iodinated by the iodogen method (Fraker and Speck, 1978) as described elsewhere (Selvaraj and Moudgal, 1993). In brief, the hormone (10 μg in 10 μL) was exposed to ^{125}I-Na (0.5 mCi/5 μL) in iodogen-coated tube for 3 min on ice, and the mixture passed through a Sephadex G-50 column to separate the free ^{125}I-Na from the hormone-incorporated ^{125}I. Specific activity of the radiolabeled hormone was between 4×10^4 and 5×10^4 cpm/ng hCG.

RRA Procedure

The assay was carried out in PBS containing 0.1% BSA and 1 m*M* PMSF. Receptor membrane preparation (80–100 μg/100 μL) was added to duplicate tubes containing the hCG standard (1.9–500 mIU/tube) in 100 μL buffer or 200 μL of serum sample, and the volume made up to 0.5 mL with the buffer. For nonspecific binding, 100 IU hCG were included. Generally, the receptor preparation was added at the end. The tubes were mixed well and preincubated at 37°C for 15 min in a shaking water bath. At the end of the preincubation period, ^{125}I-hCG (100,000 cpm/100 μL) was added to all the tubes, and incubation continued for a further 1 h. The assay was terminated by adding 1 mL of ice-cold buffer followed by centrifugation at 1600*g* for 20 min. The pellet was washed twice with the buffer, and the radioactivity associated with the final pellet was measured in an LKB Gamma Counter (Selvaraj and Moudgal, 1993).

Immunoassay

The samples were analyzed by a commercially available conventional monoclonal-based enzyme immunoassay (EIA) using standards calibrated against the NIBSC standard 2nd ISP 80/52 (Enzymun-Test® LH, Boehringer Mannheim GmbH Diagnostic, Germany). This assay is highly specific to LH with no measurable crossreactivity with FSH, TSH, or hGH.

Serum Samples

Blood samples were obtained with the informed consent of patients visiting the clinic for a periodic health checkup. The samples were left at 4°C for 24 h, and the serum separated was stored at –20°C until used for the assay. Nine normal female and six adult normal male samples were analyzed. The pregnant females (n = 3) were in their first trimester of pregnancy. The menopausal women (n = 20) were in the age group of 44–60 yr.

Progesterone and Protein Assays

Progesterone in the medium was determined by radioimmunoassay (Erlanger et al., 1959; Kohen et al., 1975; Amsterdam et al., 1979). Protein concentration in the membrane preparation was measured by the Bradford method (Bradford, 1976).

Statistical Analysis

The bioassays were calculated with Riacalc program using weighted Logit-Log regression analysis (LKB-Wallace, Turku, Finland). The RRA data were analyzed by a four-parameter logistic RIA program developed by D. Rodbard (NIH, Bethesda, MD). Each of the serum samples was estimated in triplicate in the bioassay. Data represent mean ± SEM of the values of different samples. The differences between the levels of serum LH obtained by the bioassays and RRA with the EIA were analyzed by paired Student's t-test.

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