NOTE

Radioreceptor Assay for Cynomolgus Monkey Serum Luteinizing Hormone

TAKASHI YOSHIDA¹, KINUE YOKOTA², FUMIAKI CHO¹ AND
SHIGEO HONJO¹

Tsukuba Primate Center for Medical Science¹, The National Institute of Health and ²The Corporation for Production and Research of Laboratory Primates Yatabe-machi, Tsukuba-gun, Ibaragi-ken 305

Abstract

Serum luteinizing hormone (LH) of cynomolgus monkeys was measured by radioreceptor assay (RRA). The assay system consisted of 100 μ l of standard or unknown samples, 100 μ l of a receptor preparation and 100 μ l of ¹²⁵I-LH (LER-960). Dispersed interstitial cells of mature rat testes were suspended in Tris-HCl buffer containing MgSO₄ (5 mM), bovine serum albumin (0.1%) and sucrose (0.3 M) and used as the receptor preparation. ¹²⁵I-LH was dissolved in the same buffer. The incubation was carried out at 37°C for 2 to 3 hr. The nonspecific inhibitory effect of sera in the radioreceptor assay system was compensated for by using LH-free diluent prepared by heat treatment of pooled sera. Validation of the assay demonstrated good reliability in terms of accuracy, precision and sensitivity (equivalent to 3.1 μ g LER-907/ml of serum). Recovery experiments were performed by adding known amounts of cynomolgus pituitary LH to the normal serum and mean recovery and the standard deviation of the mean was 86 $\pm$ 13%. The intra- and inter-assay coefficients of variation were 7 and 9%, respectively. By using this RRA system, cyclic changes in serum LH during the menstrual cycle were determined in three adult female cynomolgus monkeys. These results indicate that this RRA system is useful in measuring serum LH in female cynomolgus monkeys.

In studies on human reproduction, non-human primates serve as an excellent experimental model. However, it is unfortunate that there are hardly any assay methods for non-human primate luteinizing hormone (LH). The only useful assay system for non-human primate LH has been the heterologus radioimmunoassay (RIA) method of Niswender *et al.* (1971) that uses anti-ovine LH serum. On account of the limited availability of this system, attempts have been made to develop a sensitive bioassay

method for determining circulating levels of LH in non-human primates. One of them is the method of Wicking *et al.* (1979) which uses testosterone production by testicular Leydig cells of mice *in vitro* as the index. The radioreceptor assay (RRA) method that was originally devised for measurement of pituitary LH (Catt and Dufao, 1973) is also a powerful candidate, since a RRA method that is useful in determining plasma follicle stimulating hormone (FSH) of the rat was reported by Minegishi *et al.* (1980). We establish a RRA system for measurement of serum LH in the cynomolgus monkey

(*Macaca fascicularis*), and could demonstrate changes in the serum LH level of females during the menstrual cycle.

Materials and Methods

Preparation of LH receptor

Mature male rats of Wistar-Imamichi strain were obtained from the Imamichi Institute for Animal Reproduction, Dejima-mura, Ibaragi-ken, Japan. Immediately after the rats were decapitated, their testes were removed and weighed. All of the following procedures were performed under chilled conditions. The testes were decapsulated and dispersed in a 0.9% NaCl solution with stirring on a magnetic stirrer for 30 min and then the suspension was filtrated through double layers of cheesecloth to remove fragments of seminiferous tubules. The filtrate was centrifuged at 6,000 rpm for 15 min. The precipitate was resuspended in buffer and used as a receptor preparation. One milliliter of the receptor preparation contained cells derived from 1 g of the original testicular tissue. The buffer consisted of 40 mM Tris-HCl (pH 7.5), 5 mM MgSO₄, 0.1% of bovine serum albumin (BSA) and 0.3 M sucrose.

Hormone preparation

NIAMDD-human LH (One mg of LER-960 has a potency equivalent to 4,620 IU of LH) was used as the highly purified LH preparation for radioiodination with ¹²⁵I. A crude preparation of human LH (One mg of LER-907 contains 60 IU of LH and 20 IU of FSH) and a highly purified human FSH preparation (NIH-FSH-HS-1, one mg contains 187 IU of LH and 4,990 IU of FSH) were used as competitors for the binding of the radioactive hormone. The serum LH concentration was expressed in terms of LER-907.

Hypophyseal extract

An adenohypophysis of a mature female cynomolgus monkey (29 mg in wet weight) was dehydrated in cold acetone and dried *in vacuo*. The dried gland (9.0 mg) was homogenized in 0.9 ml of buffer. After centrifugation of the homogenate at 6,000 rpm for 15 min, the supernatant fluid was collected, divided into many aliquots and stored at -80°C. It was used as a LH preparation of the cynomolgus adenohypophysis.

Iodination of LH

The iodination of NIAMDD-human LH (LER-960) with ¹²⁵I was performed by using lactoperoxidase as the catalyst (Ishii and Farner, 1976). Then, radioiodinated hormone (¹²⁵I-LH) was separated from inorganic iodide by column-chromatography on Sephadex G-75. The specific activity of the labeled LH was estimated to be 8 mCi/mg. Dilution of ¹²⁵I-LH was done with assay buffer.

Radioreceptor assay system

The RRA was carried out by the method of Ishii and Adachi (1977). The assay system consisted of 100 μ l of an unknown or standard sample solution, 100 μ l of the receptor preparation, and 100 μ l of a ¹²⁵I-LH solution with about 14,000 cpm of radioactivity in a disposable tube. The mixture was then incubated at 37°C for 2 to 3 hr with shaking (120 cycle/min). At the end of incubation, 1.0 ml of chilled buffer was added to each reaction tube, and the tube was centrifuged at 6,000 rpm for 4 min at 4°C. The precipitate was washed twice with chilled buffer by repeating resuspension and centrifugation. The final sediment was counted for the radioactivity in an automatic gamma spectrometer (Model PRIAS, Packard Instru. Comp. Inc.).

Preparation of LH-free diluents

Three types of diluents which were practically LH free were prepared. To prepare the first type, sera were collected from 64 normal female monkeys and each of them was assayed for LH with the RRA method mentioned above. Three of the 64 sera contained undetectable amounts of LH even at the sample volume of 300 μ l per tube and they were used as practically LH free sera. The second type diluents were prepared as follows: a pooled serum collected from a large number of normal female monkeys was used. It was divided into five glass tubes with 5 ml in each, and then four of them were heated in a boiling water bath for 2, 5, 10 and 20 min respectively, and the remaining one (a control serum) was not heated. Immediately after heating, each serum was chilled and the coagulum was stirred vigorously. After centrifugation of the coagulum at 10,000 rpm for 2 hr, a clear supernatant fluid of heat-treated serum (SHTS) was obtained. The last type diluents were prepared as follows: sera were collected separately from pregnant and non-pregnant females and heated in a boiling water bath for 10 min. SHTSs were obtained by the

same method as mentioned above.

Radioimmunoassay of 3', 5'-monophosphate (cyclic AMP)

The *in vitro* bioassay was carried out essentially as previously described (Mendelson *et al.*, 1975). Rat Leydig cells (1×10^6 cells) in 490 μ l of Medium 199 (GIBCO) containing 22 μ g of 3-isobutyl-1-methylxanthine (Sigma) and 10 μ l of cynomolgus serum sample was incubated, then the concentration of cyclic AMP accumulated in the reaction medium was measured by the RIA.

Experimental animals

Cynomolgus monkeys captured in South East Asia were used. Mature female monkeys were housed in individual cages under indoor conditions with a room temperature of $25 \pm 2^\circ\text{C}$, relative humidity of $60 \pm 5\%$, light and dark cycle of 14L10D and fresh air changes 10 times per hr. Every day they were fed with *ca* 100 g of commercially-prepared monkey chows, *ca* 100 g of apples and oranges. These animals were estimated to be more than five years old by examining the dental formula. Menstrual bleeding was daily observed and regular menstrual cycles were confirmed all the year round. Blood samples were withdrawn from the femoral vein under anesthesia with Ketamin-HCl. The samples were centrifuged at 3,000 rpm for 15 min. Sera were separated and stored at -80°C until assayed.

For the determination of serum LH concentrations at different days of the normal menstrual cycle, three female monkeys were employed. Blood samples were collected most days at

1500. For 4 days around the expected day of ovulation (Yoshida *et al.*, 1982), blood samples were collected three times a day at 0900, 1500 and 2100.

Statistic analysis

Relative potency estimates and their 95% confidence limits were calculated by the method of Bliss (1952) and statistical analyses for lineality and parallelism were performed according to Sakuma (1964). For the determination of the minimum effective dose of the assay, the least-significant-difference method was employed.

Results

Effects of supernatant fluids from heat-treated sera added the RRA system of binding of $^{125}\text{I-LH}$

This experiment was conducted in order to know the optimal duration of heat-treatment for obtaining the LH-free SHTS under an assumption that boiling destroys receptor binding activity of LH in serum without changing noncompetitive binding affecting activity of it. $^{125}\text{I-LH}$ in 100 μ l of buffer, 100 μ l of the receptor preparation and 100 μ l of the SHTSs heated for 0 to 20 min were mixed and incubated. In order to determine nonspecific binding, the pituitary extract equivalent to 25 μ g of LER-907

Table 1. Effects of duration of the heat-treatment (100°C) of a pooled normal cynomolgus monkey serum added to the reaction mixture on total and nonspecific binding of $^{125}\text{I-LH}$ to rat Leydig cells *in vitro*.

Duration of heat-treatment (min)	Total binding		Nonspecific binding	
	(%)		(%)	
0 (Control)	4.59	3.97	1.65	1.16
2	6.95	7.35	1.56	1.50
5	7.73	8.02	1.70	1.67
10	8.13	8.27	1.85	1.67
20	8.08	7.96	1.80	1.60

Values were obtained from duplicate assays.

n.s. not significant

* $p < 0.05$

** $p < 0.01$

Table 2. Total and nonspecific binding of ^{125}I -LH to rat Leydig cells *in vitro* in the presence of the supernatant fluids from heat-treated sera of pregnant and nonpregnant monkeys.

Monkeys	Number of individuals	Mean total binding $\pm$ S.D. (%)	Mean nonspecific binding $\pm$ S.D. (%)
Nonpregnant	7	7.42 ± 0.55	1.94 ± 0.12
Pregnant	8	8.05 ± 0.57	1.88 ± 0.08

Fig. 1. Standard curves obtained by using cynomolgus pituitary extract dissolved in HTS prepared for 10 min by heating (●-●), dissolved in normal serum with an undetectable LH level (○-○) and serum from pregnant monkey diluted in normal serum with an undetectable LH level (△-△). Incubation was performed at 37°C for 2 hr.

was diluted with corresponding serum and used. As shown in Table 1, the heat-treatment increased the total binding, while it had no significant effect on the nonspecific binding (Table 2). The effect was dependent on the heating duration but reached the maximum at 10 min, showing no more enhancing effects at 20 min.

The effect of the heat-treatment for 10 min was compared between sera of nonpregnant and pregnant (4 to 20 weeks of gestation) cynomolgus (Table 2). Levels of both total and nonspecific binding did not differ significantly ($p > 0.05$) between pregnant

and nonpregnant monkeys. The binding levels were also similar to those observed in the sera treated for 10 min or more in the first experiment.

Comparison of the competition curve between an intact serum containing a large amount of LH and two types of substitute diluents with the pituitary extract

This experiment was designed in order to examine whether the SHTS and sera with an undetectable amount of LH can be used as diluents of the serum and pituitary samples for the LH assay or not. The pituitary

extract was diluted serially with the SHTS heated for 10 min of normal serum and one of sera with an undetectable amount of LH. Serum of pregnant cynomolgus monkey (3 weeks of gestation) was also diluted serially with the normal serum with an undetectable LH level. They were all used as competitors of the binding of ^{125}I -LH. Three competing curves thus obtained had an approximately common B_0 level (binding with no competitor) and also a common non-specific binding level (Fig. 1). Furthermore, they were approximately parallel with each other. The minimum effective dose of the pituitary, represented as the minimum dose that showed a statistically significant difference ($p < 0.01$) from the B_0 level by the least-significant-difference method (Sakuma, 1968), was $0.31 \mu\text{g}$. This dose was equivalent to 20 mIU. The serum of the pregnant monkey was estimated to contain LH activity equivalent to a 10.5 (7.4–14.7) IU/ml mean with a 95% confidence limit of the assay.

Comparison of competitive binding potencies of different primate gonadotropin preparations

Competitive binding potency was compared among the following primate gonadotropin preparations: highly purified human LH (LER-960), a crude human LH preparation (LER-907), highly purified human FSH (NIH-FSH-HS-1) and acetone-dried cynomolgus monkey pituitaries (Fig. 2). As expected from the purity, LER-960 was most potent and the dose required for 50% inhibition of the specific binding (total binding minus nonspecific binding) was about 10 ng per tube. LER-907 was 60 times less potent than LER-960. Acetone-dried pituitary powder was as potent as LER-907. NIH-FSH-HS-1 showed slight inhibiting potency at dose levels over $0.156 \mu\text{g}$ per tube.

Intra- and inter-assay coefficient of variation and recovery

The mean inter- and intra-assay varia-

Fig. 2. Comparison of dose-response curve between cynomolgus pituitary extract (●—●), human LH preparation, LER-907 (□—□), highly purified human LH preparation, LER-960 (○—○) and highly purified human FSH preparation, NIH-FSH-HS-1 (△—△) in LH-RRA. RRA was carried out on system consisting of standard or sample solution (100 μl), receptor preparation (100 μl) and ^{125}I -LH (100 μl). Incubation was performed at 37°C for 3 hr.

Table 3. Accuracy test on recovery of LH to normal serum from a female cynomolgus monkey.

LH added (μg^{**})	LH measured* (μg^{**})	Recovery* (%)
0	0.46 ± 0.11	—
0.5	0.85 ± 0.11	89 ± 11
1.0	1.23 ± 0.20	84 ± 14
2.0	2.08 ± 0.31	85 ± 14
Mean		86 ± 13

* Mean $\pm$ S.D.

** LH activity is expressed as LER-907 equivalent.

Fig. 3. Correlation between serum LH concentration determined by LH-RRA and stimulation of cyclic AMP accumulation in isolated rat Leydig cell. Horizontal and vertical bars indicate 95% confidence limit of LH-RRA and cyclic AMP-RIA, respectively.

Fig. 4. Typical pattern of serum LH levels during menstrual cycle in the cynomolgus monkey. Vertical bars indicate 95% confidence limits of the assay. The open bars indicate menses, dotted lines and open circles indicate undetectable levels in RRA and serum containing LH lower than the level, respectively.

tions estimated from the results of 10 consecutive assays, using 1.25 and 2.5 μg of the acetone-dried pituitary powders dissolved in the heat-treated serum as the sample, were 7 and 9 per cent, respectively. The index of precision (λ) was 0.05 ± 0.02 (mean $\pm$ S.D., $n=20$). The recovery rate for LH activity added to the normal serum in the present assay system was studied. When 0.5 to 2.0 μg of acetone-dried powder of the cynomolgus monkey hypophysis was assayed as the LH sample added to the normal female serum, the recovery rate ranged from 84 to 89%, and the mean was 85% (Table 3). The standard deviation of the recovery data was 13%.

Correlation between RRA and bioassay

LH activity data for four different sera were compared between the present RRA and the biological assay using the change in the cyclic AMP concentration in isolated rat Leydig cells as the index (Mendelson *et al.*, 1975). As shown in Fig. 3, activities estimated by these methods were well correlated, $r=0.921$, and statistically significant ($p<0.05$).

Serum LH levels during a menstrual cycle determined by the RRA

Changes in serum LH levels during a menstrual cycle were studied in three cynomolgus monkeys by the present RRA method (Fig. 4). A conspicuous LH surge was detectable on day 10 of the cycle in all the three individuals. The serum level of LH at the time of the surge was between 10 and 20 $\mu\text{g}/\text{ml}$ in terms of LER-907. Between days 5 and 9 of the cycle, the LH level was as low as the non-detectable level of the system or just on the marginal level. The second inconspicuous surge or a gradual rise in the LH level was observed from day 12 of the cycle.

Discussion

The usefulness of the application of gonadal receptors to the *in vitro* assay of gonadotropin was pointed out by Catt and Dufau in their review (1973). An RRA system for quantification of monkey and human pituitary LH has already been reported (Leidenberger and Reichert, 1972, 1973). However, these studies were carried out with pituitary preparation or purified human chorionic gonadotropin (hCG) as the sample. When we attempted to apply the RRA method to the assay of serum samples, nonspecific interference by the serum was a serious problem. Saxena *et al.* (1974) reported a RRA system for measuring the serum LH and hCG using bovine corpora lutea as the receptor preparation, but they mentioned nothing about the nonspecific interference by serum. In our preliminary experiments, a potent nonspecific interference with the binding of ^{125}I -LH to the receptor preparation was demonstrated in the monkey serum, resulting in a flattening of the standard curve. The total binding was about 10% or more of the radioactivity bound to the receptor without the influence of the serum and reduced to 6% in the presence of serum with an undetectable LH level (Fig. 1). Minegishi *et al.* (1980) established another RRA system for measuring rat plasma FSH employing the FSH-free serum as the compensator for plasma sample obtained by an immunoadsorbent column chromatography. They further employed merthiolate to remove the nonspecific interfering effect of plasma. In the present study, we employed a heat-treatment technique in order to eliminate endogenous LH activity. This technique did not affect the nonspecific interfering activity of normal sera in our assay system. Regardless of whether the donor was pregnant or non-pregnant, heated sera showed the same level of nonspecific interference.

Furthermore, normal serum with an undetectable LH level was quite as useful as the heated sera, since there was no significant difference in the total binding, non-specific binding levels or slopes in the inhibition curves obtained with all of these sera as diluent of the sample. The competitive inhibition experiment with human LH and FSH preparation clearly show that this system is sensitive to LH but not to FSH. A slight inhibition with NIH-FSH-HS-1 at high dose levels may be due to contamination by a slight amount of LH in this preparation. The potency ratio of 60 between LER-907 and LER-960 estimated in the present RRA coincided well to the potency ratio of 77 provided by the NIAMDD. The strong correlation ($r=0.921$) between the results of the RRA and bioassay with the cyclic AMP production also indicates the reliability of the RRA method.

In the accuracy test, although practically the same recovery rate was obtained with three different doses of LH added, the recovery rate itself (86%) was not satisfactory enough (Table 2). An improvement in our assay system in this point is required.

In the last experiment in which serum LH levels of three female cynomolgus monkeys were measured, the time of the LH surge was the 10th day of the menstrual cycle in all three cases. In cynomolgus monkeys, the present authors estimated from the distribution of the days of the FSH surge that the day of ovulation was approximately between the 11th and 14th days after menstruation (Yoshida *et al.*, 1982). An FSH surge was observed one or two days prior to ovulation, so the LH surge observed on the 10th day of the cycle coincided with the case of the FSH surge. In general, the fluctuating patterns of circulating LH levels during the normal menstrual cycle in the cynomolgus monkey determined by the RRA evidently resemble those obtained in rhesus monkeys and the ring-tailed lumur by the RIA (Yamaji *et al.*, 1973, Hodgen *et al.*,

1976, Norman *et al.*, 1978), and those obtained in the rhesus monkey by the *in vitro* bioassay method (Puri *et al.*, 1980). However, the values for serum LH levels in the cynomolgus monkey reported here are about ten times higher than those obtained in the rhesus monkey by the RIA (Niswender *et al.*, 1971). In rhesus monkeys, basal serum LH levels were overestimated by the RIA and peak serum LH levels were underestimated by the RIA as compared to the results obtained by the bioassay (Neill *et al.*, 1977, Dufau *et al.*, 1977). The reason why these differences occurred is not yet clear. The authors suggest the following two possibilities: one is that the rat Leydig cell LH receptor has a higher affinity with cynomolgus monkey LH than with human LH, and the other is that the cynomolgus monkey has a higher serum LH level than the other monkeys. It would be interesting to examine serum samples of monkeys other than the cynomolgus monkey with the present RRA system.

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