Determination of biologically active LH in the serum of male rhesus monkeys (*Macaca mulatta*)

E. J. Wickings, M. H. Qazi* and E. Nieschlag

*Abteilung Experimentelle Endokrinologie, Universitäts-Frauenklinik, Westring 11,
D-4400 Münster, Federal Republic of Germany*

Summary. The in-vitro bioassay for LH, using mouse Leydig cells, has been modified for the direct measurement of serum LH in the male rhesus monkey. Validation of the assay demonstrated good reliability in terms of accuracy, precision and sensitivity (1.5 mIU/ml). Basal LH concentrations in laboratory-maintained monkeys with and without anaesthesia were not significantly different from those in free-ranging, feral monkeys. LH-RH (50 µg i.v.) elicited a 30-fold increase in LH concentrations after 30 min. LH levels in castrated adult monkeys were approximately 50 times the normal levels. Intact and castrated juvenile males had only very low LH levels. LH from the serum of an adult male castrate was further characterized by Sephadex G100 column chromatography.

The in-vitro bioassay provides a preferable alternative to the heterologous radioimmunoassay method for the routine determination of LH in the rhesus monkey.

Introduction

Serum LH concentrations in the female rhesus monkey have been characterized in detail by radioimmunoassay (see Knobil, 1974, for review), but very few data on LH in the male monkey have been published (Mori & Hafez, 1973; Toivola, Bridson & Robinson, 1978). The most commonly used radioimmunoassay system has been the 'ovine-antiovine' system (Niswender *et al.*, 1971) which showed considerable species cross-reactivity for LH (Niswender, Midgley, Monroe & Reichert, 1968; Niswender, Reichert, Midgley & Nalbandov, 1969). The assay was thought to be specific for LH, but discrepancies were found when this assay was employed to measure the LH content of serum from hypophysectomized monkeys (Neill, Dailey, Tsou & Reichert, 1977).

The development of the in-vitro bioassay for LH, utilizing Leydig cell preparations from either the mouse (Van Damme, Robertson & Diczfalusy, 1974) or the rat testis (Dufau, Mendelson & Catt, 1974) has provided an opportunity of combining the sensitivity of a radioimmunoassay endpoint with the measurement of the biological activity of LH. This bioassay system has been further adapted for the determination of LH in human serum (Qazi, Romani & Diczfalusy, 1974; Graesslin *et al.*, 1976; Dufau, Pock, Neubauer & Catt, 1976) and in the serum of female rhesus monkeys (Neill *et al.*, 1977). We have modified the system utilizing mouse Leydig cells (Van Damme *et al.*, 1974) for the direct measurement of biologically active LH in untreated serum of male rhesus monkeys. LH was also measured under various physiological conditions and serum LH from an adult male castrated monkey was further characterized by column chromatography.

* Present address: Department of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan.

Materials and Methods

Preparation of the Leydig cell suspension. The in-vitro bioassay for LH was based on the original method of Van Damme *et al.* (1974), with some modifications. Two mice were killed for each preparation by cervical dislocation and the testes were immediately removed, decapsulated and cut into 8–10 pieces per testis with scissors in ice-cold Eagle's medium (EM; 4 testes/20 ml; Gibco–Biocult, Karlsruhe) containing sodium bicarbonate (0.1%), HEPES buffer (25 mM; Serva, Heidelberg), calf serum (1.0%; Gibco–Biocult) and theophylline (90 µg/ml EM; Byk Gulden, Konstanz) at a final pH of 7.4. The testis pieces were gently stirred on a magnetic stirrer for 15 min at 4°C and then filtered through a fine nylon gauze and centrifuged at 80 g for 10 min at 4°C. The pellet was resuspended in EM (20 ml) and preincubated at 35°C for 60 min, centrifuged, washed in 40 ml ice-cold EM and re-centrifuged. In order to determine the number of cells present in the incubation medium the Eagle's medium was divided as follows: the cell pellet equivalent to 15 ml of the original filtrate was taken up in 15 ml EM and stirred gently on ice until use in the assay. The pellet from the remaining 5 ml equivalent of the filtrate was resuspended in 0.5 ml Trypan blue solution (0.1%) and the number of cells/µl filtrate was counted in a Thoma cytometer at a 40-fold magnification. The preparations yielded an average of 20×10^6 cells/testis. The cell concentrations for the assay were adjusted as follows: for normal and juvenile monkeys, EM was added to give a final cell concentration of 1×10^6 cells/100 µl EM, and for the serum of the castrate and the column fractions, a final cell concentration of 500 000 cells/100 µl EM was used.

Cell incubation. The LH standard routinely employed in the assay was the human pituitary 1st International Reference Preparation (MRC 69/104), at a dose range of 30–500 µi.u. Two other pituitary preparations, the rhesus preparation LER 1909-2 and the human standard preparation LER 907, were also investigated and used at dose levels of 30–250 ng and 1.5–25 ng respectively. Serum from juvenile and normal adult monkeys was diluted with EM to give dose ranges from 2.5 to 20 µl serum/100 µl EM. Sera from adult castrates were diluted to the dose range of 16–1000 nl/100 µl EM.

Cells (100 µl) and standards or serum dilutions (100 µl) were incubated together in glass incubation tubes (12 × 75 mm) for 3 h at 35°C with shaking. The reaction was stopped by placing the tubes in a boiling water bath for 15 min. When 500 000 cells were used in the incubation, the radioimmunoassay for testosterone was performed directly in the incubation tubes. When 1×10^6 cells were used, 0.5 ml ice-cold EM was added to each tube, and 200 µl of this medium were then assayed for testosterone. Standard doses were always incubated with the same number of cells as the unknown samples. A blank value (100 µl cells + 100 µl EM) was included in every assay. All values were measured in quadruplicate.

Radioimmunoassay of testosterone. This was performed as described by Nieschlag & Loriaux (1972) without thin-layer chromatography. Following overnight incubation of the EM fraction with diluted antiserum (end dilution 1/45 000) and 10 000 c.p.m. [³H]testosterone at 4°C, the antibody-bound and free fractions were separated using dextran-coated charcoal. The standard curve for testosterone ranged from 10 to 2000 pg, and 200 µl EM was added to all standard tubes.

The testosterone produced per tube during the 3-h incubation was calculated from the testosterone standard curve following linearization of the data by log–logit transformation. A standard curve for LH was then constructed by plotting the log (LH concentration) against the square root of the testosterone produced (pg)^½, which gave a linear plot over a wide dose range (Van Damme *et al.*, 1974). The dose–response curves of the serum samples were plotted and when parallel to the standard curve the LH concentrations in the serum dilutions were read from the standard curve at 2 different serum volumes. The overall index of precision (λ) of each assay, for the LH standard curve and the serum samples, was calculated according to the method of Gaddum (1933). The detection limit of the testosterone radioimmunoassay was 13 ± 4 pg (mean

Table 1. Recovery of human LH added to serum from a normal male rhesus monkey in the in-vitro bioassay for rhesus LH

LH added (μ i.u.)	LH measured (μ i.u.)	
	0.3 μ l serum	0.66 μ l serum
0	32.5	72
66	101.0 (103.8%)	145 (110.6%)
100	137.0 (104.5%)	178 (106.0%)

± 1 s.d.; $n = 27$). The accuracy of the assay in the in-vitro conditions was checked by diluting serum with EM, incubating for 3 h without cells in the medium, boiling and measuring the testosterone present in the medium. The mean (± 1 s.d.) recovery of testosterone was $99.0 \pm 9.3\%$ ($n = 11$). The overall accuracy of the bioassay method was checked by adding known amounts of the LH standard to different doses of rhesus serum and measuring the testosterone produced during the incubation (Table 1).

Sephadex G100 column chromatography. Serum from a castrated adult male monkey (500 μ l) was chromatographed on a Sephadex G100 column (1.0×60 cm, equilibrated with 0.16 M-NaCl) and 2.2 ml fractions were collected using a LKB fraction collector (35 drops/9 min). Each serum fraction eluted from the column was monitored for LH activity in the in-vitro bioassay, 100 μ l being incubated with 500 000 cells/100 μ l EM in triplicate. The optical density of the fractions was measured at 280 nm, and the void volume of the column was calculated using dextran blue. The elution position of BSA was also determined. Ovine LH (LER-1056-C2) was iodinated using the chloramine T method (Hunter & Greenwood, 1962) and the protein-bound and free iodine-25 were separated on a Bio-Gel P60 column (0.8×20 cm) by elution with 0.01 M-phosphate-buffered saline, pH 7.5. The 125 I-labelled oLH (70 000 c.p.m.) was applied to the G100 column and the elution pattern of the iodinated material was monitored.

Text-fig. 1. Typical dose-response curves for the 3 different pituitary LH preparations (1, human LH (LER 907); 2, human LH (MRC 69/104); 3, rhesus LH (LER 1909-2)) and for dilutions of serum from an adult male castrated rhesus monkey (4). Each tube contained 500 000 mouse Leydig cells and the total testosterone produced was measured directly in the medium.

Experimental animals. Male rhesus monkeys were housed under constant laboratory conditions in single cages without access to female monkeys. Since the monkeys had been obtained from the wild, exact data on the age of the individual animals were not available and the monkeys were therefore classified according to their body weights (see Table 3). The testes had been removed from the castrated monkeys at least 2 months before these investigations. Blood samples were collected from a cubital vein from monkeys that were conscious or under ketamine anaesthesia (Ketalar: Parke-Davis, Freiburg; 8 mg/kg i.m.). LH-RH tests were carried out in 4 conscious monkeys restrained in primate chairs for the test period. Following collection of a basal blood sample between 08:00 and 11:00 h, LH-RH (50 µg in 1 ml saline: Hoechst, Frankfurt) was injected i.v. and a further blood sample was taken 30 min later.

Blood samples were also obtained from free-ranging monkeys living in family troops in northern Pakistan. These animals were anaesthetized with ketamine for blood sampling. Serum samples were stored at -20°C until analysis.

Results

Typical dose-response curves for the three pituitary preparations investigated and the dose-related response from serial dilutions of serum from an adult male castrated rhesus monkey (40–220 nl serum) are shown in Text-fig. 1. The potency estimates of 2 of the LH preparations in terms of the other are shown in Table 2. The effect of LH-RH on the dose-response curve of serum from a normal adult male rhesus monkey is illustrated in Text-fig. 2.

The LH activities indicated in Table 3 are reported in terms of the standard MRC 69/104. The detection limit of the assay when using 1×10^6 cells/100 µl and diluting the incubation

Text-fig. 2. Dose-response curves of serum from a normal male rhesus monkey before (2) and 30 min after (3) treatment with LH-RH (50 µg). The standard preparation (1) was MRC 69/104. Each tube contained 1×10^6 mouse Leydig cells and the incubation medium was diluted before assay for testosterone.

Table 2. Potency estimates of 2 pituitary LH preparations in terms of the human LH preparation MRC 69/104

	Potency (μ i.u./ng)	95% confidence limits	Index of precision (λ)
Human LH (LER 907)	2.68	2.38–2.98	0.093
Rhesus LH (LER 1909-2)	1.17	1.09–1.25	0.055

Table 3. Serum LH concentrations in male rhesus monkeys under various conditions

Animal no.	Body wt (kg)	Serum LH (mi.u./ml)	95% confidence limits	Index of precision (λ)
<i>Intact laboratory adults</i>				
2899	8.0	25.2	23.6–26.8	0.077
2892	8.1	7.3	6.7–7.9	0.042
2896	7.5	2.0	1.7–2.3	0.036
2861	8.1	17.0	16.4–17.6	0.037
788	7.0	22.9	22.7–30.1	0.050
2898	7.7	7.2	7.1–7.3	0.086
2031	9.3	29.0	28.6–29.4	0.075
1717	9.6	16.9	16.2–17.6	0.081
2781	7.5	16.5	15.8–17.2	0.064
2877	7.0	45.0	44.0–46.0	0.097
Mean $\pm$ 1 s.d.		18.9 $\pm$ 12.5		
<i>30 min after 50 μg LH-RH i.v.</i>				
2899	8.0	334	320–348	0.077
2892	8.1	350	330–370	0.040
2896	7.5	302	284–320	0.053
2861	8.1	426	416–436	0.046
Mean $\pm$ 1 s.d.		353 $\pm$ 53		
<i>Anaesthetized laboratory adults</i>				
2899	8.0	20.2	18.6–21.8	0.064
2892	8.1	18.5	17.9–19.1	0.066
2896	7.5	27.1	25.3–28.9	0.036
2861	8.1	6.8	6.0–7.4	0.032
Mean $\pm$ 1 s.d.		18.2 $\pm$ 8.4		
<i>Free-ranging adults, anaesthetized</i>				
007	12.9	14.6	14.2–15.0	0.050
021	11.1	2.8	2.2–3.4	0.050
034	11.2	11.7	11.4–12.0	0.051
037	7.6	23.5	21.9–26.1	0.045
Mean $\pm$ 1 s.d.		13.2 $\pm$ 8.5		
<i>Adult castrates</i>				
115	8.7	656	574–734	0.048
761	12.0	703	627–719	0.111
<i>Intact juveniles</i>				
779	2.5	2.2	1.8–2.6	0.043
803	4.6	2.0	1.8–2.2	0.077
796	2.3	2.2	2.0–2.4	0.069
<i>Castrated juveniles</i>				
791	4.2	2.4	2.0–2.8	0.083
793	2.2	2.8	2.2–3.4	0.036
797	2.3	1.8	1.6–2.0	0.050

medium was 1.5 mi.u./ml. In the laboratory monkeys, basal LH values measured 10 min after the start of ketamine anaesthesia were not significantly different from basal values in conscious animals, and the concentrations in the anaesthetized free-ranging monkeys were also similar. Intravenous administration of 50 μ g LH-RH elicited an approximately 30-fold increase in serum LH concentrations after 30 min, but the values were not as high as those found in 2 male adult castrated monkeys. The LH levels were very low in intact and castrated juvenile animals.

The elution patterns of serum from a castrated adult male monkey and 125 I-labelled ovine LH (LER 1056-C2) are shown in Text-fig. 3. The elution position of rhesus LH corresponded to that of ovine LH, with V_e/V_0 values of 1.32 and 1.41 respectively ($V_0 = 12.8$ ml). BSA was eluted before both LH peaks ($V_e/V_0 = 1.18$). The absorbance profile of the chromatographed serum showed that the peak of LH activity was eluted at a position where the majority of the plasma proteins had already been eluted from the column.

Text-fig. 3. Sephadex G100 elution patterns of serum from a castrated male rhesus monkey (—) and of 125 I-labelled ovine LH (---). The absorption profile at 280 nm (····) is also shown.

Discussion

The lack of suitable purified standard preparations and antisera has limited the development of radioimmunoassay systems for the determination of LH in non-human species. One attempt at overcoming this problem has been the use of heterologous radioimmunoassays, such as the 'ovine-antiovine' system for LH (Niswender *et al.*, 1968), showing wide species cross-reactivity. This assay has been applied to the measurement of hypophysial and circulating LH in the rhesus monkey (Niswender *et al.*, 1971), but doubts have been expressed about the validity of this method (Neill *et al.*, 1977). When compared to LH values measured by the rat in-vitro bioassay method of Dufau *et al.* (1976), the radioimmunoassay data failed to detect the magnitude of changes in LH concentrations, indicating only the trend in serum levels (Neill *et al.*, 1977). The availability of an in-vitro bioassay for LH, whether using mouse or rat Leydig cells, provides an opportunity of measuring biologically active LH with a sensitivity unattainable in the classical in-vivo bioassay methods. The LH values obtained from the in-vitro bioassay must be

considered as more reliable than those given by radioimmunoassay (Qazi *et al.*, 1974; Romani, Robertson & Diczfalusy, 1976; Neill *et al.*, 1977).

The validation of the in-vitro bioassay for LH has proved that this method shows greater specificity for LH than the heterologous radioimmunoassay (Van Damme *et al.*, 1974), and also has the advantage of being applicable to the measurement of LH in many species (Farmer, Suyama & Papkoff, 1977; Neill *et al.*, 1977). We have characterized the mouse Leydig cell bioassay for the direct measurement of LH in the serum of the male rhesus monkey. Previous applications of this method to the measurement of LH in serum had involved purification of the serum by gel filtration and isoelectric focusing before the in-vitro bioassay of LH content (Qazi *et al.*, 1974; Romani *et al.*, 1976). In the method described here, the assay is performed without prior treatment of the serum. No interfering serum factors could be detected, as seen from the results of the additivity studies performed. This technique has been validated for the detection of any possible non-specific serum effects by van Damme *et al.* (1974) and Romani *et al.* (1976). In addition we have demonstrated good reliability criteria in terms of precision, accuracy and sensitivity.

The design of the assay, however, has the consequence that the method is less practical than the corresponding radioimmunological method. One technician can analyse 45 samples per week, measuring all sera at 3 dose levels, and all values being determined in quadruplicate. Since the method is based on bioassay techniques, at least a 2 + 2 point assay design is essential. Some authors, however, have not adopted this design, thereby jeopardizing the value of the method (Dufau *et al.*, 1976; Neill *et al.*, 1977).

While it is difficult to compare the LH concentrations measured here with existing data in the literature, because of methodological differences, the results obtained under various conditions appear physiologically plausible. The 30-fold increase in serum LH after LH-RH treatment proves conclusively that the pituitary of the rhesus monkey is capable of responding to the releasing hormone, and that the magnitude of the response is far greater than that measured by radioimmunoassay (Spies & Niswender, 1973; Ferin, Warren, Dyrenfurth, Vande Wiele & White, 1974; Spies & Norman, 1975; Levitan, Beitins, Milton, Barnes & McArthur, 1977). Serum LH concentrations measured under ketamine anaesthesia were not different from values in conscious monkeys. Hence the values in the free-ranging monkeys, obtained under ketamine anaesthesia, can be considered as true basal values, indicating that captivity conditions do not affect LH concentrations. The failure of castration before puberty to increase LH levels, in contrast to post-pubertal castration which resulted in extremely high LH levels, demonstrates the inactivity of the 'gonadostat' before puberty.

The data presented here for the male monkey and those of Neill *et al.* (1977) for the female suggest that the in-vitro bioassay for LH provides an acceptable and reliable means of monitoring serum LH concentrations in the rhesus monkey. Since no suitable homologous radioimmunoassay is available, this method offers a preferable alternative for the routine determination of RhLH.

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