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Radioimmunoassay for Rhesus Monkey Gonadotropins[1] (38876)

CHARLES FAIMAN,[2] ERIC L. STEARNS, JEREMY S. D. WINTER,[3] FRANCISCO I. REYES, AND WILLIAM C. HOBSON

The University of Manitoba, Departments of Physiology, Paediatrics, and Obstetrics and Gynaecology, Winnipeg, Canada, and the International Center for Environmental Safety, Holloman AFB, N.M.

The development of radioimmunoassays for rhesus monkey (*Macaca mulatta*) gonadotropins has been limited by the general lack of purified hormones for labeling and the production of antibodies. Thus, while homologous[4] methods for the assay of rhesus LH (rhLH) (1) and rhFSH (2) have been described, these reagents are not readily available.

Hormones from certain species may show usable cross-reactivity in homologous radioimmunoassay systems designed for other species: e.g., rat and rhesus LH may be measured in an ovine system (3, 4), and chimpanzee FSH and LH in human systems (5). However, it has been our experience (6 and unpublished data), and that of others (7), that rhesus gonadotropins show little, if any, cross-reactivity in most homologous human gonadotropin assays.

Midgley *et al.* (8) suggested that in this situation heterologous assays might offer two advantages: (1) they would avoid the necessity for preparing purified antigens of the species in question, and (2) they might provide better specificity among similar glycoprotein hormones. In fact, such heterologous assays have been described for rhFSH (2, 9) and multispecies FSH (10).

We describe herein heterologous radioimmunoassay methods for rhesus gonadotropins employing reagents that are readily available to investigators: purified rat or ovine gonadotropins for trace, anti-human gonadotropin antisera, and a partially purified rhesus pituitary gonadotropin standard.

Materials and Methods. The assay reagents are described in Table I. The assays protocol is essentially as previously described for human FSH (11) and is outlined in Table II.

1. *Iodination of antigen.* One to two micrograms were iodinated with ^{131}I by the chloramine-T method of Greenwood *et al.* (12) to specific activities of 80–220 $\mu Ci/\mu g$. Separation of protein-bound from free ^{131}I was done by chromatography on a Bio-gel P10 column eluted with barbital buffer (0.07 *M*, pH 8.6). It was found necessary to purify the LH trace further by chromatography on Biogel P100 (see Fig. 1) because of the presence in some assays of nonparallelism between standard and serum samples.[5]

2. *Assay conditions.* (a) *Choice of antibodies.* It must be emphasized that only two of eight anti-hFSH and one of 10 anti-hCG or anti-hLH antisera proved usable. This was ascertained by trial-and-error binding and displacement studies. (b) *First antibody dilution.* This was chosen so that 0.1 ml of antibody would bind 20–35% of trace. (c) *Timing of first stage.* In order to optimize assay sensitivity, a 24-hr preincubation of standards and unknowns with antibody, prior to addition of trace, was employed.

3. *Samples,* Peripheral venous blood samples were obtained from four animals during the course of one menstrual cycle; in two (nos. 730 and 3430) this was during the infertile time of year (May–June) and in

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[2] Reprint requests to Dr. C. Faiman, Health Sciences Centre (G-449), 700 William Avenue, Winnipeg, Manitoba, Canada, R3E 0Z3.

[3] Queen Elizabeth II Scientist.

[4] Homologous defines a radioimmunoassay system in which trace, standard, and the antigen for raising the antiserum are all derived from the same species; heterologous indicates a radioimmunoassay system in which hormone antigens from diverse species are used.

[5] Occasional batches of trace (LER-1056-C2) were found to show this phenomenon, especially if the material for iodination had previously been thawed and refrozen.

TABLE I. REAGENTS EMPLOYED FOR RHESUS GONADOTROPIN ASSAYS.

Assay	Trace	Antibody	Standard
FSH	Rat[a]	1 Guinea pig anti-human FSH[d] (GP 202B4) 2 Rabbit anti-human FSH[b] (N.P.A. 3)	Rhesus pituitary LER-M-907D[c]
LH	Ovine[e] (LER-1056-C2)	Guinea pig anti-human hCG[f] (GP1B2)	Rhesus pituitary LER-M-907D[c]

[a] From rat FSH N.P.A. kit, prepared by Dr. A. Parlow.
[b] From human FSH N.P.A. kit 3, prepared by Dr. W. D. Odell.
[c] Kindly provided by Dr. L. E. Reichert, Atlanta, GA, and containing 0.26 NIH-FSH-S1U/mg and 0.025 NIH-LH-S1 U/mg.
[d] Prepared in this laboratory against partially purified human pituitary FSH.
[e] Kindly provided by Dr. L. E. Reichert, Atlanta, GA.
[f] Prepared in this laboratory against commercial hCG (A.P.L. Ayerst).

TABLE II. REAGENTS AND CONDITIONS EMPLOYED FOR DOUBLE-ANTIBODY rhFSH AND rhLH RADIOIMMUNOASSAY[a]

Preincubation (24 hr)	Standards and unknowns diluted to 1 ml in 1% BSA-barbital (0.07 *M*, pH 8.6)		
	+		
	0.1 ml antibody—appropriate dilution to bind 20–35% of trace		
	FSH method 1	FSH method 2	LH method
	GP anti-hFSH (202B4) 1:3000	R anti-hFSH (N.P.A. 3) 1:2000	GP anti-hCG (1B2) 1:10,000
	+	+	+
Incubation (48–72 hr)	rFSH-^{131}I (0.1–0.3 ng/0.1 ml)	rFSH-^{131}I (0.1–0.3 ng/0.1 ml)	oLH-^{131}I (0.1–0.3 ng/0.1 ml)
	+	+	+
Precipitation (48–72 hr)	RAGPS	GARGG	RAGPS
	+	+	+
	Carrier NGPS	Carrier NRS	Carrier NGPS

[a] Abbreviations used: BSA-bovine serum albumin, GP-guinea pig, h-human, R-rabbit, r-rat, o-ovine A-anti, S-serum, G-goat, GG-gamma globulin, N-normal, N.P.A.-National Pituitary Agency.

two (824 and 1371) this was during the fertile time of year (October–November). In addition, two females were sampled pre- and postovariectomy. The serum was frozen at −20° until analysis.

Results. Validation of assays. Assay performance characteristics are shown in Table III. Serial dilutions of sera from eugonadal and castrate monkeys showed dose-response slopes which paralleled that of the LER-M-907D pituitary standard in both the FSH and LH systems. Recovery of standard added to 0.2 ml eugonadal serum samples averaged near 100% for each system.

The limited availability of rhesus gonadotropin preparations with varying FSH/LH ratios precluded stringent assessment of cross-reactivity. The fractions tested in both the FSH and LH systems are shown in Table IV. In the FSH assays, there was no de-

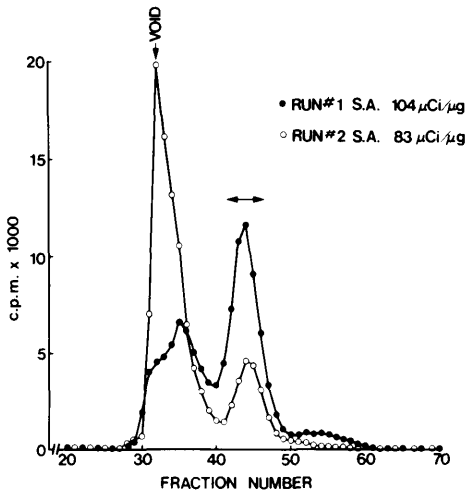

FIG. 1. Two chromatograms of ovine LH (LER-1056-C2) labeled with ^{131}I by the chloramine T method (12). Five tenths milliliter of the protein peak tube after iodination was layered on a 40 × 2.5-cm Biogel P-100 column and eluted with 1% BSA–barbital buffer, 0.07 *M*, pH 8.6. Fractions contain 1.5 ml each. The vertical arrow indicates the blue dextran void volume of the column. The retarded peak material, indicated by the horizontal arrow, was used for assay purposes and avoided the problem of nonparallelism between standard and unknowns in the assay.

tectable activity of rhCG in either system. The only serious discrepancy was with regard to the highly purified LH fraction WDP-X-81-1720 (also rich in TSH). This fraction gave similar results in both assay systems and these in turn are comparable to the results obtained by a previously published radioimmunoassay method (9). Boorman *et al.* (9) provided evidence based upon electrophoretic studies which indicate that this discrepancy is due to the presence of a small amount of material which migrates in an identical fashion to intact FSH and which is immunologically reactive but biologically inert.[6]

Application of the assays in physiologic studies. A comparison of serum results from the two different FSH assay systems is shown in Fig. 2. The expected rise in FSH levels after castration and the typical menstrual cycle patterns (2, 13) are seen. Despite the fact that immunologic potencies of pituitary fractions were comparable by both systems (Table IV), it is apparent that serum estimations are disparate, with assay 1 systematically giving higher results than assay 2. Nevertheless, the parallelism between the two sets of values is striking. Regression analysis of 72 serum samples, ranging from 250–4900 ng/ml in assay 2, revealed a high degree of correlation between the two FSH assays ($r = 0.991$, $P < 0.001$) and the following equation: Assay 2 = 16.94 + 0.699 (Assay 1). The y intercept was not significantly different from zero; thus, the values for assay 2 are approximately 70% those for assay 1 throughout the range.

The serial FSH and LH data throughout four menstrual cycles—two ovulatory and two anovulatory as assessed by serum progesterone levels are shown in Fig. 3. The ovulatory cycles are characterized by two FSH peaks, one during the early proliferative phase and a second peak coincident with the mid-cycle surge in LH. In animal 1371, which became pregnant, the second LH peak is due to cross-reactivity in the assay by CG. No consistent gonadotropin patterns were observed in the anovular cycles. FSH levels were near the limit of assay detectability in animal 3430.

In addition, LH levels were determined in animal 3385 (see Fig. 2 for FSH data). Values rose from a mean of 490 ng/ml prior to castration to 1830 ng/ml at 39 days after castration.

Discussion. We have described heterologous radioimmunoassay methods for the measurement of rhFSH and rhLH. The latter assay is also capable of measuring rhCG (see Table IV and Fig. 3, animal 1371). It should, of course, be emphasized that only certain antisera directed against human hormones can be used in these systems. However, the FSH reagents—rat FSH for labeling and anti-human FSH (N.P.A. 3, system 2) are currently available from the National Pituitary Agency and crude rhesus pituitary extract can be used for the assay standard. Similar approaches for the assay of rhesus

[6] If one assumes that pure rhFSH, like pure human FSH, has a biological potency of approximately 100 NIH-FSH-S1 U/mg (11), then even if the overestimation of purified LH (Table 4) represents true cross-reactivity, it would only amount to <2% on a weight basis.

TABLE III. RHESUS MONKEY GONADOTROPIN RADIOIMMUNOASSAY CHARACTERISTICS.

	FSH		LH
	Assay 1	Assay 2	
Dose range[a, b]	25–500 ng	25–500 ng	75–1200 ng
Minimum detectable level[b, c]	15–25 ng	10–15 ng	30–70 ng
Dilutional effect of serum	Parallel (0.05–0.4 ml)	Parallel (0.05–0.3 ml)	Parallel (0.05–0.4 ml)
Recovery from serum	102 ± 4% (SD) (n = 6)	96 ± 6% (SD) (n = 5)	100 ± 6% (SD) (n = 7)
Precision: Intrassay	8%[d]	9%	5%
Precision: Interassay	15%[d]	14%	14%

[a] In nanograms per assay tube.

[b] Expressed in terms of rhesus pituitary gonadotropin fraction LER-M-907D, with biopotencies of 0.26 NIH-FSH-S1 U/mg and 0.025 NIH-LH-S1 U/mg.

[c] Defined as 95% of initial binding (B/Bo).

[d] Coefficient of variation.

TABLE IV. CROSS-REACTIVITY OF GONADOTROPINS IN FSH AND LH ASSAY SYSTEMS AND COMPARISON WITH PUBLISHED DATA.

	FSH				LH	
		Immunoassay potency[b]				
Fraction	Bioassay potency[a]	Assay 1	Assay 2	Literature (9)	Bioassay potency[a]	Immunoassay potency[b]
LER-M-907D[c]	0.26	0.26	0.26	0.26	0.025	0.025
WDP-X1-2-A	1.10	0.86	1.09	1.02	0.048	0.043
WDP-X1-81-1720	0.10	1.84	1.70	1.55	2.90	1.60
WDP-rhCG	—	<0.24	<0.06	—	—	2.89[d]

[a] Data provided by donors of fractions and expressed as NIH-S1 U/mg (LER, Dr. L. E. Reichert; WDP, Dr. W. D. Peckham).

[b] Data expressed as NIH-S1 U/mg except for rhCG expressed as U/10,000 IU.

[c] Used as assay standard.

[d] Dose-response slope parallel to assay standard—1mIU (hCG equivalent) equals 11.6 ng LER-M-907D.

FSH (2, 9) and CG (14) have recently been reported. Moreover, the present FSH systems can also be used to measure FSH in other species, the ovine for example (see also 10).

We also attempted to use the National Pituitary Agency rat–rat FSH kit directly to estimate rhFSH. Assay of pituitary fractions gave potency estimates comparable to the two heterologous systems reported herein, but assay sensitivity was inadequate to detect circulating gonadotropin concentrations in the majority of eugonadal samples. We also attempted to use a purified ovine FSH for trace in the FSH system (instead of rat FSH trace) but the binding to the human antisera was inadequate and this approach was abandoned.

The assays are directly applicable to the estimation of circulating rhesus gonadotropin levels. Values and patterns of gonadotropins during the ovulatory menstrual cycle and after castration are similar to previously published reports (Table V). It is of interest that the two animals studied during May–June were anovular whereas the two studied during October–November were ovular as judged by gonadotropin patterns, progesterone levels, and pregnancy in one instance (see Fig. 3). These observations help to explain our experience, as well as that of others (15), that the rhesus monkey in

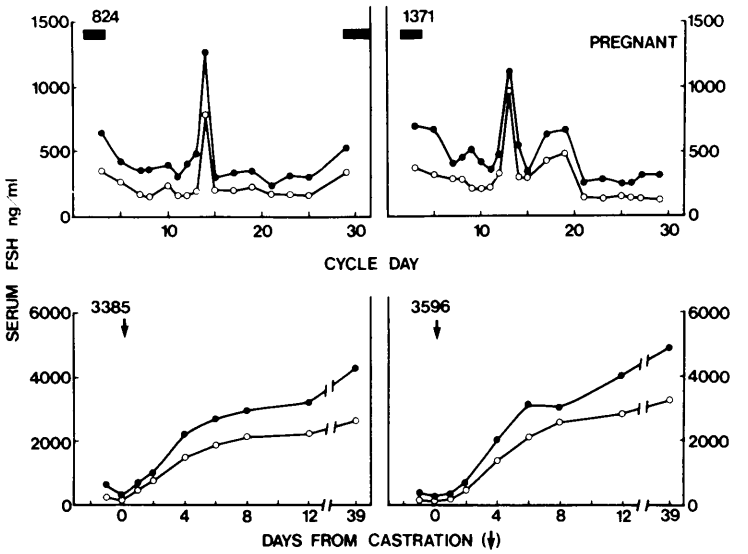

FIG. 2. Comparison of serum FSH values obtained by the two different FSH assays (● = assay 1; ○ = assay 2) reported as ng LER-M-907D standard/ml serum. The upper panels depict ovulatory menstrual cycle data; one animal (1371) became pregnant. Solid bars indicate menses. The lower panels depict the response to castration indicated by the vertical arrow.

TABLE V. COMPARISON OF SERUM GONADOTROPIN CONCENTRATIONS BY PRESENT AND PREVIOUSLY PUBLISHED METHODS[a]

	FSH				LH		
Condition	Present 1	Present 2	Boorman *et al.* (9)[b]	Yamaji *et al.* (2)[b]	Present	Monroe *et al.* (1)[c]	Niswender *et al.* (4)[d]
Menstrual cycle follicular phase	461 ±31	254 ±18	314 ±20	800–2700	327 ±24	340 ±30	297 ±18
Menstrual cycle luteal phase	397 ±46	268 ±36	209 ±15	800–2400	474 ±37	380 ±70	228 ±15
Menstrual cycle ovulatory peak	1180	895	1243 ±256	5400	1720	4000 ±800	1024 ±142
Castrate	4600	2950	—	—	2100	3300 ±400	1180

[a] Values expressed as mean ± SE (or range) in ng equivalents LER-M-907D/ml serum. Where different standard used (1, 2), conversion to above units based upon biopotency data provided.

[b] Heterologous system.

[c] Homologous system.

[d] Ovine — ovine system.

North America is infertile during the spring and summer months and fertile during the fall and winter.

Although there was good agreement between the two different FSH assay methods for estimations of potency of pituitary extracts (Table IV), a systematic bias was observed for estimations of circulating FSH levels (see Figure 2), with values obtained in assay 2 being approximately 70% of those obtained in assay 1. The ratio of assay 2/assay 1 values was constant throughout a wide range of values—from eugonadal to castrate. Thus, had any one serum been used as the assay standard, the values obtained with the two systems would have been virtually identical. The exact mechanism for this bias in serum, but not

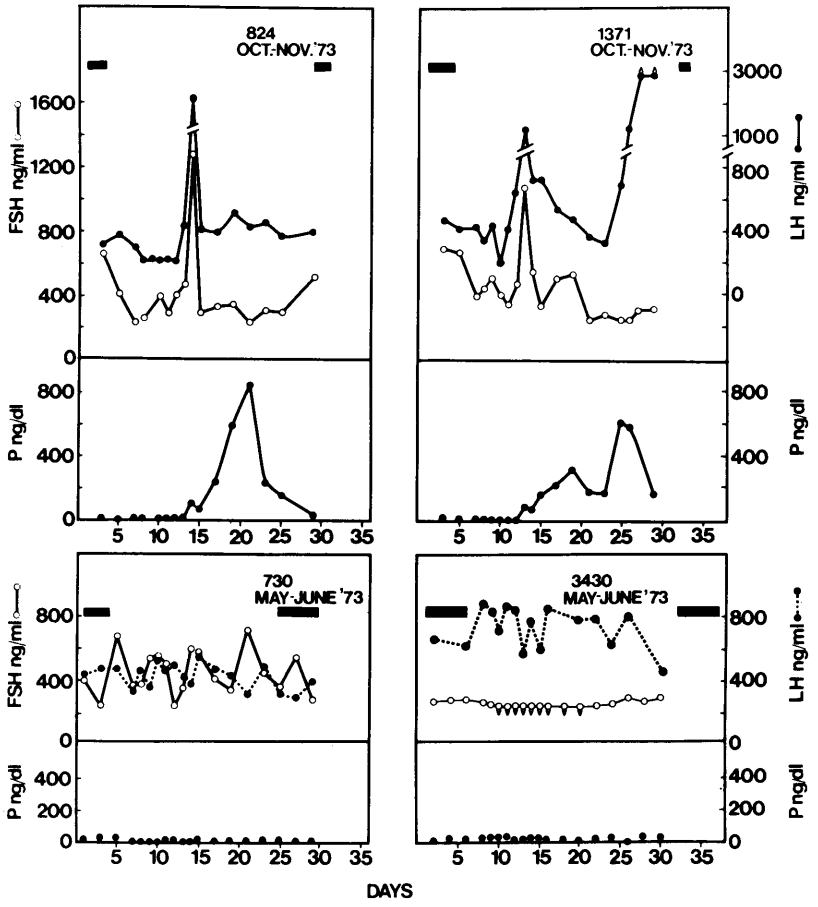

FIG. 3. Serum FSH (assay 1), LH and progesterone (P) levels (measured by radioimmunoassay) during four menstrual cycles—two anovulatory (below) and two ovulatory (above)—one resulting in pregnancy. Bars represent menses. $\wedge$ - greater than. V = less than. Gonadotropin values expressed as ng LER-M-907D standard/ml serum.

pituitary specimens, remains unexplained. The findings do suggest that: 1) circulating FSH is not identical to extracted pituitary FSH; and 2) that the two antisera recognize a slightly different portion of the FSH molecule. A similar bias due to antisera has also been reported for human gonadotropin assay systems (16). These findings underscore the need for assay standards which are as similar as possible to the unknowns being estimated (17).

Summary. Heterologous double-antibody radioimmunoassay methods are described for the measurement of circulating levels of rhesus monkey (*Macaca mulatta*) FSH and LH; the latter assay is also applicable to rhesus chorionic gonadotropin (CG) estimations. The FSH assay utilizes purified rat FSH for trace, either of two anti-human FSH antisera and a semipurified rhesus pituitary standard. The LH assay utilizes purified ovine LH for trace, an anti-human CG antiserum and the same rhesus pituitary standard. The use of these systems obviates the necessity of purifying rhesus gonadotropins which are required for the development of homologous radioimmunoassay systems.

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