Insensitivity of Bonnet Monkeys to (D-Ala^6, Des-Gly^{10}) LHRH Ethylamide, a Potent New Luteinizing Hormone Releasing Hormone Analogue in Rats and Mice

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ABSTRACT. The Luteinizing Hormone Releasing Hormone (LHRH) activity of (D-Ala^6, Des-Gly^{10}) LHRH ethylamide was compared with that of LHRH in oöphorectomized bonnet monkeys by determining serum LH and FSH concentrations at various time intervals after a sc injection of 100 μg of LHRH and either 100 μg or 1 mg of the analogue. Following administration of synthetic LHRH, a significant rise in both serum LH and FSH was observed. In contrast, no discernible change in serum gonadotropin concentrations was noted following injection of the analogue (D-Ala^6, Des-Gly^{10}) LHRH ethylamide, previously reported to have greatly increased potency in rats and mice. (*Endocrinology* **100:** 918, 1977)

THE ELUCIDATION of the amino acid sequence of Luteinizing Hormone Releasing Hormone (LHRH) has made possible the design and synthesis of a variety of analogues with agonistic or antagonistic function to the parent compound. The increased biological activity of certain agonists has been attributed to a number of factors: increased affinity for receptor cells, greater efficacy to promote secretion after binding, and resistance to degradation by plasma and tissue peptidases. One analogue, (D-Ala^6, Des-Gly^{10}) LHRH ethylamide, with substitutions at the six and ten positions, has been shown to have enhanced and prolonged LH- and FSH-releasing activity in rats and mice (1–6).

It seemed particularly desirable to extend these observations to the catarrhine monkeys, whose disappointing refractoriness to synthetic LHRH detracts from their usefulness as models for the human reproductive cycle (7–13). The species studied was the bonnet macaque, whose menstrual cycle resembles that of the rhesus monkey and crab-eating macaque (14,15). The subjects were oöphorectomized in order to maximize the observed response (13), allowing at least three weeks following surgery for the gonadotropins to rise (16). Since in the rat the greater potency of the analogue had been best manifested following subcutaneous injection (4), this route of administration was selected.

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Materials and Methods

1. *Subjects*

Adult female bonnet monkeys (*Macaca radiata*) were individually caged and fed monkey chow and fresh fruit. Two monkeys had been previously oöphorectomized, and four other monkeys were oöphorectomized at a minimum of three weeks prior to study. Serum samples for gonadotropin measurements were obtained prior to and three weeks following oöphorectomy (Table 1).

2. *Peptides*

The peptides LHRH and (D-Ala^6, Des-Gly^{10}) LHRH ethylamide were obtained from Beckman Instruments, Inc. Both were diluted with sterile saline and individual doses of 100 μg/.05 ml were quick frozen in separate vials. Each vial was thawed immediately preceding an experiment.

3. *Experimental design*

All monkeys were sedated with phencyclidine (Sernylan®) at an initial dose of 2 mg im (.5 mg/kg) for immobilization and placement of an iv catheter. Subsequently they were kept comfortably restrained by an immobilizing blanket without additional anesthesia. Our laboratory had previously verified the lack of effect of phencyclidine on baseline or LHRH-stimulated gonadotropin levels, as shown by Ferin *et al.* (12,17). The saphenous vein was then percutaneously catheterized, and a heparin lock inserted (#16 or #18 Intracath®, 10,000 U/l, flush every 15 min).

All six monkeys received both LHRH and the analogue (D-Ala^6, Des-Gly^{10}) LHRH ethylamide in separate trials in randomized order, at approximately three week intervals. At the commencement of each trial, control samples of serum were drawn (t = −30, −15, 0 min), the average gonadotropin concentration of which provided the baseline value. A sc injection of 100 μg of LHRH or the analogue was given in 1 cc saline at t = 0 min. Venous sampling was continued for 4 h (t = 15, 30, 45, 60, 90, 180, 240 min). Four of the six monkeys then received an additional trial at the 1 mg dose of the analogue. In these experiments the serum sampling was extended over 8 h (t = 300, 360, 420, 480 min as well). The total blood loss of 40–60 cc was well tolerated by all subjects. An im injection of Imferon® (2 cc = 50 mg Fe) was given at the conclusion of each trial.

The serum was separated and kept frozen at −20 C until assayed.

4. *Radioimmunoassay*

Gonadotropins were measured by a modification of the heterologous double antibody radioimmunoassays of Niswender *et al.* (18,19). Applicability of the heterologous system to the measurement of serum LH in the bonnet monkey had previously been demonstrated (14,15). We applied it as well to the FSH assay, not hitherto utilized for the bonnet monkey. Ovine LH (LER 1056) and rat FSH were iodinated with ^{125}I by the chloramine-T method, then purified by passage over Sephadex G-100. Antibodies were a rabbit anti-ovine preparation GDN #15 provided by G. D. Niswender and used at a final dilution of 1:512,000, and a rabbit anti-human FSH obtained from the National Pituitary Agency and used at a dilution of 1:15,000. The reference standard was rhesus monkey pituitary LER-M907D, the gift of L. E. Reichert, Jr. Results were expressed in ng/ml of this standard. Standard curves were based on eight replicates for each of eight points. Samples were assayed at 100 μl for LH, and 200 μl diluted 1:4 for FSH. Figure 1 (A and B) shows the parallelism of the standard curve for LH and FSH with a single control sample of serum in serial dilution. All samples were determined in triplicate in a single assay, the intraassay coefficient of variation being 5.0% for LH, 2.9% for FSH.

TABLE 1. Pre- and post-oöphorectomy concentrations of serum gonadotropins (expressed as ng/ml LER-M907D) in the bonnet monkey

Subject		Pre-oöphorectomy (ng/ml)	Post-oöphorectomy (ng/ml)
1	LH	422	6,249
	FSH	302	3,358
2	LH	382	3,867
	FSH	201	3,072
3	LH	367	5,674
	FSH	322	3,650
4	LH	1,997	2,112
	FSH	2,823	2,567

Results

The response of serum gonadotropins to the sc injection of LHRH is shown in Fig. 2(A and B), expressed as the mean % change in LH and FSH, respectively, from the pre-injection baseline. Determination of the mean regression of both gonadotropins for the six monkeys revealed a highly significant rise ($P < 0.001$). For LH, the maximum serum concentration was achieved at 180 minutes. For FSH the mean slope was more shallow, and the time course longer, so that the concentration may have still been increasing at 240 min.

Of note was the considerable variation in LHRH responsiveness between the subjects. On an individual basis, the LH response was significant for five of the six monkeys, whereas only the two subjects showing the greatest LH response had a significant rise in FSH.

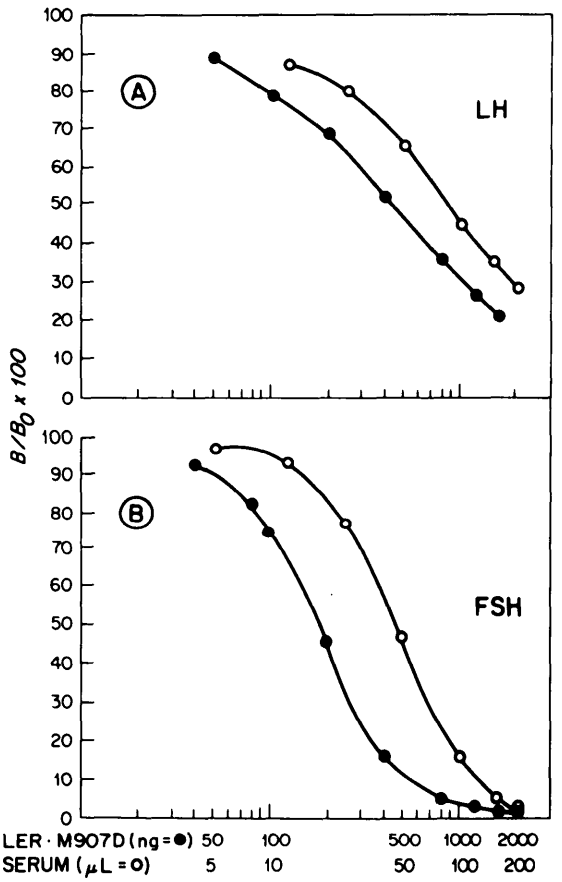

FIG. 1. Dose-response curves for LER-M907D and bonnet monkey serum in a heterologous LH (1A) or FSH (1B) radioimmunoassay. Bo = bound at zero concentration.

As can be seen in Fig. 2 (C–F), no significant change in the mean serum gonadotropin levels occurred following injection of 100 μg or 1 mg of the analogue (D-Ala^6, Des-Gly^{10}) LHRH ethylamide. Even for the individual monkeys which showed the greatest response to synthetic LHRH, the regression coefficients of the gonadotropin response to the analogue were not significant.

An incidental finding of this study was the tenfold increase in serum FSH concentration which followed oöphorectomy (Table 1). This paralleled a rise in serum LH concentration similar to that previously reported for the rhesus monkey. One monkey (Subject 4), who had ceased cycling and was thought to be post-menopausal, showed elevated gonadotropins prior to surgery.

Discussion

The monkey is widely used for the study of reproductive physiology due to the similarity of its menstrual cycle to that of the human. In particular, this animal has provided a model system in which to study the mechanisms of gonadotropin control. Therefore, it has been disappointing that, while the administration of synthetic LHRH has been shown to release gonadotropins in many species including man, the monkey has exhibited a relative insensitivity. Spies and Niswender failed to observe release of LH in the rhesus monkey following iv injection of synthetic LHRH, although they did elicit a response with ovine stalk-median eminence extract (7,8). They re-

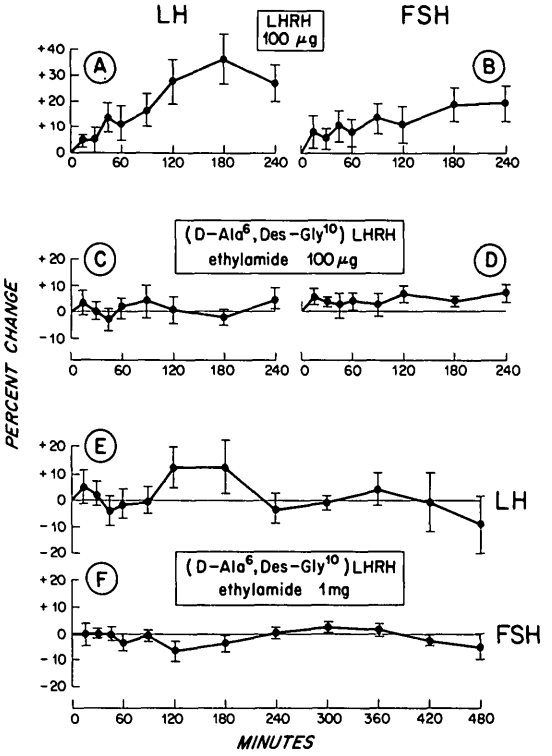

FIG. 2. Mean (± 1 SEM) per cent change in serum gonadotropins in response to LHRH 100 μg (2A,2B), (D-Ala^6, Des-Gly^{10}) LHRH ethylamide 100 μg (2C,2D), and (D-Ala^6, Des-Gly^{10}) LHRH ethylamide 1 mg (2E,2F) injected sc at t = 0 min.

ported a response to both compounds after intrapituitary infusion (7). Arimura *et al.* found that LHRH only occasionally increased serum LH in the rhesus monkey, using various modes of administration (9). Mori and Hafez also described a variable rise in the crab-eating macaque (10). Ehara *et al.* reported no response to iv LHRH administration in the rhesus macaque (11). Ferin *et al.* and Krey *et al.* both studied the LHRH response of the rhesus monkey at different stages in the ovarian cycle, and found the greatest effect of synthetic LHRH by iv injection and infusion to occur at the time of the normal LH surge (12,13). However, the small and short-lived increments in circulating LH and FSH elicited by even the largest doses of LHRH never approached the magnitude of spontaneous or estrogen-induced gonadotropin surges. Our results in the bonnet macaque confirm those in the rhesus and crab-eating macaques, in that the gonadotropin response to LHRH varied greatly among individual monkeys, and was quantitatively much smaller for even the most responsive subjects than the LH and FSH release seen in other species. The discrepancy between these *in vivo* studies and the significant and dose-related LH release demonstrated in monolayer cultures from monkey anterior pituitary cells following incubation with synthetic LHRH suggests the importance of additional factors not present in the *in vitro* system (20).

The analogue (D-Ala^6, Des-Gly^{10}) LHRH ethylamide has been reported to have a greatly increased potency in several *in vivo* and *in vitro* systems. In ovulation induction in the diestrous rat, the analogue showed an increased potency, in comparison to LHRH, by a factor of 50 to 80 (1), while in the proestrous rat pretreated with fluphenazine dihydrochloride to block ovulation, the potency of the analogue was approximately 36 times that of synthetic LHRH (2). In the immature male rat, a 4 h iv infusion showed LH-releasing activity of 1600% and FSH-releasing activity of 1200% that of LHRH; similarly, sc administration to this animal produced a 31-fold greater increase in LH and a 9-fold greater increase in FSH, over a 6 h period, than that found for LHRH (3,4). Although the potency was only 2 to 3-fold that of LHRH in the hemisected rat pituitary (1), the ED_{50} of this analogue in rat pituitary cell cultures was reported as 30 times that of synthetic LHRH (5). In the diestrous mouse as well, the analogue was found to be more effective in induction of ovulation than the parent compound (6). However, in the monkey, contrary to our anticipation, this compound appeared to have no discernible effect. The reason for this discrepancy in the relative potency of the LHRH analogue between species is not apparent. It has been postulated that the refractoriness of the monkey to exogenously administered LHRH is attributable to its rapid degradation by an undetermined factor in the plasma or pituitary cell cytoplasm. Such inactivation may be even more efficient for the analogue. Alternatively, there may be a real interspecies difference of the pituitary cell receptor and the degree to which modifications in the structure of LHRH augment the cell's secretory response.

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Addendum

The potency of the same lot of (D-Ala^6, Des-Gly^{10}) LHRH ethylamide was verified in a human subject.

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The Commission of Biochemistry of the Union of Chemical Societies of Yugoslavia and the International Society of Neuroendocrinology will cosponsor a Meeting on "The Receptors and Metabolism of Steroid Hormones in the Structures of the Central Nervous System—Biochemistry, Pharmacology and Clinical Uses." The Symposium will be held in Zagreb, Yugoslavia on May 27–29, 1977.

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