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F. Garza
LSU Agricultural Center

D. L. Thompson
LSU Agricultural Center

D. D. French
LSU Agricultural Center

J. J. Wiest

R. L. St George

See next page for additional authors

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Active Immunization of Intact Mares against Gonadotropin-Releasing Hormone: Differential Effects on Secretion of Luteinizing Hormone and Follicle-Stimulating Hormone

FRANK GARZA, JR., D. L. THOMPSON, JR.,[1] D. D. FRENCH,[2] J. J. WIEST, R. L. St. GEORGE, K. B. ASHLEY, L. S. JONES, P. S. MITCHELL, and D. R. McNEILL

Departments of Animal Science and Veterinary Science[2]
Louisiana Agricultural Experiment Station
Louisiana State University Agricultural Center
Baton Rouge, Louisiana 70803

ABSTRACT

Five lighthorse mares were actively immunized against gonadotropin releasing hormone (GnRH) to determine the relative importance of this hypothalamic hormone in the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Five mares immunized against the conjugation protein served as controls. Mares were initially immunized in November and received secondary immunizations 4 wk later, and then at 6-wk intervals until ovariectomy in June. All mares immunized against GnRH exhibited an increase ($p<0.01$) in the binding of tritiated GnRH by plasma, an indication that antibodies against this hormone had been elicited. Concentrations of LH, FSH and progesterone in weekly blood samples were lower ($p<0.05$) in GnRH-immunized mares than in controls after approximately 4 mo of immunization. However, the LH concentrations were affected to a greater degree than were FSH concentrations. All five control mares exhibited normal cycles of estrus and diestrus in spring, whereas no GnRH-immunized mare exhibited cyclic displays of estrus up to ovariectomy. All mares were injected intravenously with a GnRH analog (which cross-reacted <0.1% with the anti-GnRH antibodies) in May, after all control mares had displayed normal estrous cycles, to characterize the response of LH and FSH in these mares; two days later, the mares were injected with GnRH. The LH response to the analog, which was assessed by net area under the curve, was lower ($p<0.01$) by approximately 99% in mares immunized against GnRH than in control mares. In contrast, the FSH response to the analog was similar for both groups. The FSH response to GnRH in mares immunized against GnRH was 85% lower than that in control mares ($p<0.05$), an indication that the antibodies against GnRH did prevent much of the injected GnRH from reaching the pituitary. At ovariectomy, combined ovarian weights of GnRH-immunized mares were 57% lower than those of control mares ($p<0.01$); numbers of follicles and corpora lutea were also lower ($p<0.01$) than those of controls. It was concluded that LH and to some degree FSH secretion, and probably LH production, in the mare are dependent upon the bioavailability of GnRH. However, the lack of effect of GnRH-immunization on FSH response to the analog indicates that FSH stores in the pituitary (and perhaps FSH production) are relatively independent of GnRH bioavailability.

INTRODUCTION

In horses, concentrations of luteinizing hormone (LH) in the blood appear to be tightly coupled in some physiologic states to concentrations of follicle-stimulating hormone (FSH). For example, gonadectomy results in increased concentrations of both gonadotropins in blood (Ginther, 1979; Thompson et al., 1979); administration of gonadotropin-releasing hormone (GnRH) results in a rapid increase in concentrations of both hormones (Ginther, 1979; Thompson et al., 1979, 1983d); and spontaneous, simultaneous peaks in concentrations of both hormones have been reported for both mares and stallions (Thompson et al., 1983a, 1986).

Under other conditions, usually involving gonadal steroids, concentrations of LH and FSH in blood appear to be independent. For example, LH concentrations are stimulated by administration of estrogen to gonadectomized horses whereas FSH concentrations are suppressed (Ginther, 1979; Thompson et al., 1979, 1983d; Garza et al., 1985). Moreover,

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[1] Reprint requests: Donald L. Thompson, Jr., Department of Animal Science, Louisiana State University, Baton Rouge, LA 70803.

FSH production is increased in ovariectomized mares treated with testosterone propionate whereas LH production is not affected (Reville-Moroz et al., 1984).

Another condition under which LH and FSH concentrations in blood differ is seasonal anestrus. Hart et al. (1984) reported that the amount of LH in pituitaries from anestrous mares in winter was only 15% of that in pituitaries of mares in the breeding season. In contrast, pituitary content of FSH did not differ due to season. These results agree well with secretion rates of LH and FSH after administration of GnRH to anestrous mares (approximately 94 times more FSH than LH; Thompson et al., 1986). Hart et al. (1984) suggested that the reduced LH content in the pituitaries of mares in the nonbreeding season was due to the lowered hypothalamic content (and presumably secretion) of GnRH that they found in those same mares.

Given that pituitary content and secretion rates of FSH differ markedly from those of LH during winter, we hypothesized that there is a component of FSH production and/or secretion in the horse that is relatively independent of GnRH's reaching the pituitary. Thus, the present experiment was designed to determine the long-term effects of active immunization of intact mares against GnRH on concentrations of LH and FSH in blood and on their relative responses to exogenous secretagogues.

MATERIALS AND METHODS

Ten lighthorse mares (≥2.5-yr-old) that had exhibited normal estrous cycles during the previous breeding season were used. All mares were kept on pasture and fed grass hay as needed to maintain good body condition throughout the experiment. On November 1, five randomly selected mares were immunized with 4.0 mg of a conjugate prepared from GnRH and bovine serum albumin (BSA) as described by Fraser et al. (1974). The remaining five mares were immunized with 4.0 mg of BSA (controls). The primary immunizations were prepared in Freund's complete adjuvant. Immunizations consisted of multiple intramuscular injections in the neck and/or hip regions of each mare. Secondary immunizations (2.0 mg antigen), similarly in Freund's incomplete adjuvant, were given 4 wk after the primary immunizations, and then every 6 wk thereafter until ovariectomy. Samples (10-ml) of jugular blood were collected from each mare immediately before the primary immunization and then weekly throughout the experiment. The ovaries of all mares were evaluated by rectal palpation once a month for size (length, width and depth) during March, April and May.

Beginning February 1, all mares were checked daily for estrus by exposure to a stallion. After all control mares had exhibited at least two normal estrous cycles in the spring (a normal estrous cycle was defined as at least 3 consecutive days of estrus followed by at least 8 consecutive days of diestrus), each control mare was randomly paired with one GnRH-immunized mare for the timing of all subsequent events. This was because no GnRH-immunized mare exhibited normal estrous cycles during the late winter and spring. Thus, for the following procedures (GnRH-analog administration, GnRH administration, and ovariectomy), one control mare and one GnRH-immunized mare were treated at the same time based on the stage of the control mare's estrous cycle.

On Day 8 or 9 of diestrus in May, one jugular vein of each mare was fitted with a 14-gauge catheter for frequent blood sampling. Three 10-ml samples of blood were collected from each mare at 15-min intervals. Each mare was then administered a GnRH analog (des-Gly^{10}-(im-Bzl-D-His^{6})-LHRH; Sigma Chem. Co., St. Louis, MO) through the jugular catheter at 40 ng/kg of body weight. Blood samples were collected at 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min after the injection of analog. The GnRH analog was chosen because it was found in preliminary trials to cross-react <0.1% with the antibodies in the plasma of these mares when tested with tritiated GnRH. The dosage of GnRH analog was also determined in a preliminary trial (with different mares) and was equivalent to 1.0 μg/kg of body weight of GnRH with regard to LH and FSH response (area under the curve).

Forty-eight h after the injection of GnRH analog, the above procedure was repeated for each mare except that GnRH (Sigma) was injected. The dose of GnRH was 1.0 μg/kg of body weight.

On Day 7 or 8 of the subsequent diestrus (in June), each pair of mares was bilaterally ovariectomized via flank incision under local anesthesia. Each ovary was trimmed of connective tissue, weighed, and serially sectioned (approximately 6-mm sections) for the enumeration of follicles and corpora lutea.

For all blood samples, heparinized plasma was harvested via centriguation and stored at −15°C. Concentrations of LH, FSH and progesterone were

measured by radioimmunoassay as described previously (Thompson et al., 1983a,b,c). The binding of tritiated GnRH to diluted plasma was measured and the cross-reactivities of various GnRH analogs (Sigma) were determined as described previously for estradiol (Thompson and Honey, 1984).

Data for measurements taken over time were analyzed by analysis of variance that accounted for the repetitive nature of the sampling (split-plot design; Gill and Hafs, 1971). Differences between groups for each time period were assessed for significance by the lsd-test. Net areas under the LH and FSH response curves for GnRH and analog injections were calculated as described by Thompson and Nett (1984). Data for nonrepetitive variables were analyzed by one-way analysis of variance (Steel and Torrie, 1960).

RESULTS

The binding of tritiated GnRH by 10 μl of plasma from control and GnRH-immunized mares is presented in Figure 1. After the third injection of antigen in January, GnRH binding increased ($p<0.01$) in plasma of GnRH-immunized mares relative to control mares. There was an increase in binding after each immunization thereafter such that binding was gradually increasing up through June 6 when blood sampling ceased.

The cross-reactivities of several commercially available GnRH analogs were as follows (%): (Gly-OH^{10})-LHRH 12.9 ± 7.8; des-Gly^{10},(D-Leu^{6})-LHRH 0.28 ± 0.24; des-Gly^{10},(D-Trp^{6})-LHRH 0.31 ± 0.23; des-Gly^{10},(im-Bzl-D-His^{6})-LHRH 0.097 ± 0.054; and (Ac-D-pCl-Phe^{1,2}, D-Trp^{3}, D-Arg^{6}, D-Ala^{10}) - LHRH 0.078 ± 0.046. Because up to 5 μg of most of these analogs did not inhibit the binding of tritiated GnRH by 50%, the above cross-reactivities were calculated at the greatest percentage of inhibition obtained with a given analog. The amount of unlabeled GnRH that resulted in 50% inhibition averaged 3.92 ± 0.92 ng.

Concentrations of progesterone, LH and FSH were all suppressed ($p<0.05$) in GnRH-immunized mares relative to control mares during the latter periods of the experiment (Fig. 1). Concentrations of all three hormones in control mares varied due to the stage of the estrous cycle as evidenced by the large fluctuations in values in Figure 1. In contrast, hormonal concentrations in GnRH-immunized mares became relatively constant after approximately 15 wk of treatment.

From February 1 until ovariectomy in June, no GnRH-immunized mare exhibited cyclic displays of estrus (Table 1). In fact, during that time, only one GnRH-immunized mare exhibited estrus, and that was on two separate occasions (March 21 and May 16) for one day only. This mare did not exhibit any rise in LH or progesterone concentrations in association with these two displays of estrus. All control mares exhibited normal estrous cycles (Table 1) with an average duration of estrus of 6.4 ± 0.7 days and an average duration of diestrus of 17.3 ± 0.7 days.

During March, ovarian volume, as assessed by rectal palpation, did not differ ($p>0.05$) between the two groups of mares (Table 1). In April and May, ovarian volumes of GnRH-immunized mares averaged only 18 and 8%, respectively, of control values ($p<0.05$). At ovariectomy, ovarian weights, number of follicles >10 mm in diameter and number of corpora lutea were all lower ($p<0.01$) in GnRH-immunized mares than in control mares (Table 1).

When mares were administered GnRH analog in May, the mean concentrations of FSH before injection were lower ($p<0.05$) in GnRH-immunized mares than in control mares (Fig. 2). However, the FSH response to GnRH analog, as assessed by the change in hormonal concentrations and by the net area under the curve, was similar for both groups. In contrast, when

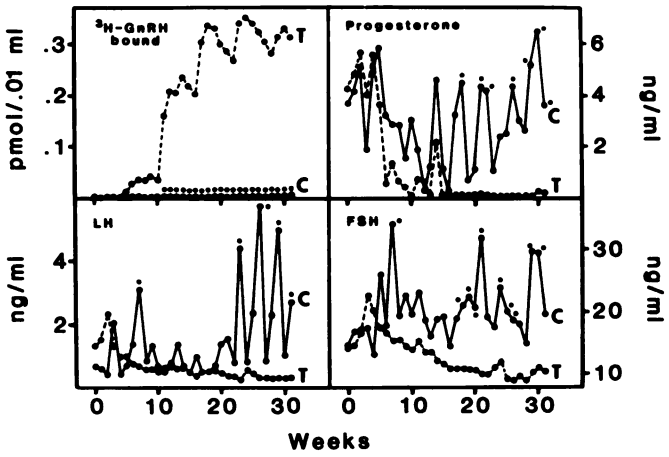

FIG. 1. Binding of tritiated GnRH and concentrations of progesterone, LH, and FSH in plasma of control mares (C; *solid lines*) and GnRH-immunized mares (T; *dasbed lines*). *Asterisks* indicate differences ($p<0.05$) between groups for the indicated period. The primary immunizations (Week 0) were given on November 1, and the final samples (Week 31) were drawn on June 6. Pooled SEM from the analyses of variance were 0.016 pmol and 1.26, 1.16 and 3.55 ng/ml for GnRH-binding and progesterone, LH, and FSH concentrations, respectively.

TABLE 1. Estrous and ovarian characteristics of control and GnRH-immunized mares before and during the breeding season.

Characteristic	Group	
	Control	GnRH-immunized
Estrous cycles/mare[a]	3.6 ± 0.8[b]	0**
Ovarian volume (cm^3)[c]		
March	117 ± 38	64 ± 14
April	189 ± 47	35 ± 5*
May	640 ± 262	54 ± 7*
Ovarian weight (g)[d]	140 ± 23	60 ± 4**
Follicles >10 mm/mare[d]	11.6 ± 3.1	0**
Follicles ≤10 mm/mare[d]	10.6 ± 2.5	9.2 ± 2.3
Corpora lutea/mare[d]	1.4 ± 0.2	0**

[a]From February 1 to ovariectomy in June.

[b]Mean ± SEM.

[c]Sum of both ovaries estimated via rectal palpation (length × width × depth).

[d]At ovariectomy in June.

*$p<0.05$.

**$p<0.01$.

GnRH itself was administered (Fig. 2), the FSH response was reduced ($p<0.05$) in GnRH-immunized mares compared with control mares.

The LH response to GnRH analog (Fig. 3) was essentially nonexistent in GnRH-immunized mares relative to control mares ($p<0.01$). The same lack of response was observed for LH after administration of GnRH. For both LH and FSH, the shape of the response curves differed for the analog and GnRH injections, with the analog resulting in a sustained elevation in concentrations through 6 h after injection.

DISCUSSION

Active immunization of these mares against a GnRH-BSA conjugate resulted in the production of anti-GnRH antibodies, as has been reported for other species (Clarke et al., 1978; Fraser and Baker, 1978; Chappel et al., 1980; Shettigara et al., 1981). Moreover, repetitive injection of antigen at 6-wk intervals resulted in high GnRH binding in plasma of GnRH-immunized mares such that the long-term effects of immunization could be determined. Although the specificity of the anti-GnRH antibodies could not be exhaustively assessed (and was not attempted), it appears from the analog data that minor deviations from the GnRH structure greatly reduced the cross-reactivity of an analog when it was in competition with GnRH. If this apparent specificity was also present in vivo, then we would conclude that only GnRH or closely related compounds in plasma were

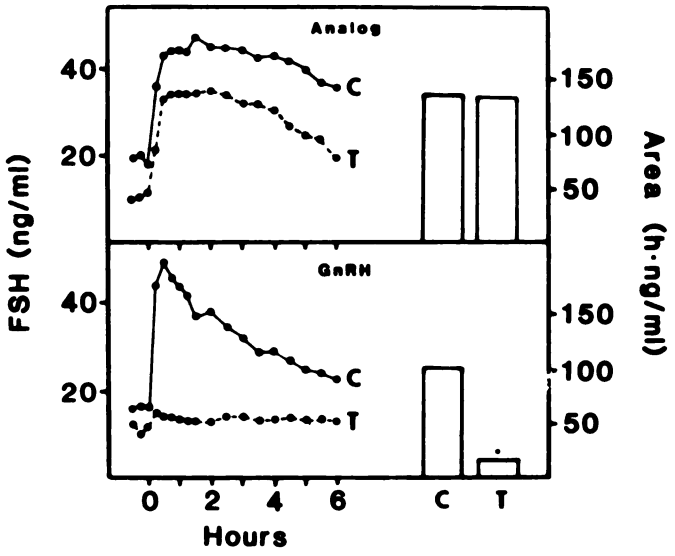

FIG. 2. Concentrations of FSH in plasma of control mares (C; *solid lines*) and GnRH-immunized mares (T; *dashed lines*) before and after an injection of GnRH analog (*top panel*) and GnRH itself (*bottom panel*). *Bar graphs* indicate net areas under the response curves. GnRH injections were given 48 h after analog injections. The *asterisk* indicates a difference ($p<0.05$) between groups. Pooled SEM from the analyses of variance were 3.9 ng/ml for FSH concentrations and 20 units for areas.

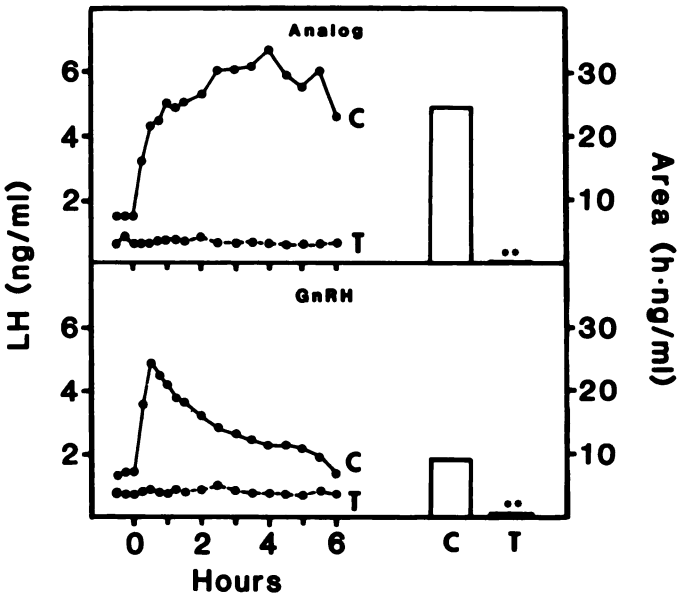

FIG. 3. Concentrations of LH in plasma of control mares (C; *solid lines*) and GnRH-immunized mares (T; *dashed lines*) before and after an injection of GnRH analog (*top panel*) and GnRH itself (*bottom panel*). *Bar graphs* indicate net areas under the response curves. GnRH injections were given 48 h after analog injections. *Asterisks* indicate differences ($p<0.01$) between groups. Pooled SEM from the analyses of variance were 0.64 ng/ml for LH concentrations and 4.5 units for areas.

affected by the antibodies generated against the GnRH moiety of the conjugate.

Because horses are seasonal breeders, the mares in the present experiment were first immunized in the autumn so that they received several secondary immunizations before the onset of the next breeding season. Our goal was to study the long-term effects of immunization on characteristics of LH and FSH secretion, thereby minimizing the importance of pituitary stores and/or turnover rates that could mask any real effects of treatment in the short term. The failure of GnRH-immunized mares to return to normal estrous cycling in the spring is similar to the cessation of normal reproductive activity observed in other species immunized against GnRH (Clarke et al., 1978; Fraser and Baker, 1978; Shettigara et al., 1981). Thus, it appears that the generation of anti-GnRH antibodies in these mares resulted in a biological inactivation of endogenous GnRH. This concept is also supported by the data in Figure 2; i.e, immunization against GnRH reduced the GnRH-induced release of FSH by 85% but did not affect analog-induced FSH release.

Concentrations of both LH and FSH in plasma were suppressed in the long term in GnRH-immunized mares relative to control mares. However, it must be noted that LH concentrations in GnRH-immunized mares were suppressed to undetectable levels (0.3 ng/ml in our assay) whereas FSH concentrations were still easily detectable after 32 wk (approximately 10 ng/ml as compared with the limit of detection of 3.9 ng/ml in the FSH assay). Thus, we conclude that normal secretion of these two gonadotropins is dependent upon GnRH's reaching the pituitary; however, there appears to be a component of FSH secretion that is relatively independent of GnRH. This is in agreement with previous observations in seasonally anestrous mares in which concentrations of plasma LH are generally undetectable whereas concentrations of FSH are not nearly as suppressed (Thompson et al., 1986). A similar dichotomy was reported for LH and FSH secretion in geldings after suppression with testosterone or estradiol (Thompson et al., 1979).

The effect of GnRH-immunization on apparent amount of hormone released in response to injection of GnRH analog differed markedly for LH and FSH. Essentially no LH was secreted by GnRH-immunized mares. In previous studies, the apparent amount of LH secreted in response to exogenous GnRH was closely coupled with the mean amount of LH in the pituitary (Thompson et al., 1979; 1986; Hart et al., 1984). If this relationship had persisted in the present experiment, the amount of LH in the pituitaries of GnRH-immunized mares would have been proportionally lower, which would be indicative of a long-term suppression of LH production. Such a suppression would be similar to that reported for LH in seasonally anestrous mares (Hart et al., 1984).

The FSH response to the injection of GnRH analog was virtually identical in both groups of mares. Again, if this analog-induced change in blood levels of FSH is indicative of pituitary stores, then we conclude that the amount of FSH in the pituitary was relatively independent of GnRH's reaching the pituitary. Because concentrations of FSH in blood in the long term were suppressed by approximately 50%, it might be expected that a concurrent and proportional reduction in FSH production would have to occur to prevent a large accumulation of FSH in the pituitary. Given that FSH secretion after injection of the GnRH analog was not indicative of such an accumulation, we conclude that some reduction in FSH production likely occurred. One possible interpretation of these data is that the production of FSH in the pituitary is coupled to the amount within the gland such that accumulation would not occur. It must also be noted that this FSH was released by the GnRH analog, thus its secretion per se was GnRH-dependent.

The overall characteristics of LH and FSH secretion in the mares immunized against GnRH were analogous to those in seasonally anestrous mares. Thus, our results agree with the speculation by Hart et al. (1984) that the low LH content in the pituitary of mares in the winter is due to the low concentration (and probably secretion) of GnRH in the hypothalamus. Moreover, continued low concentrations of LH resulted in a failure of the mares in the present experiment to return to estrus and to ovulate, which is similar to the seasonally anestrous state.

It is not known whether LH and FSH are produced by one, two or perhaps three cell types within the pituitary of the horse. In rats, at least three cell types appear to be involved with LH and FSH storage (Dada et al., 1983). This important basic information is needed to develop a model of gonadotropin secretion by the equine pituitary. The complexities of the LH vs. FSH data in the present experiment and in previous reports (Thompson et al., 1979, 1986; Hart et al., 1984; Reville-Moroz et al., 1984) tend to refute the

argument that a single cell type is responsible for both LH and FSH secretion in the horse.

In conclusion, long-term immunization against GnRH produces a reproductive state in mares analogous to seasonal anestrus. It appears that LH secretion, and probably production, in mares is highly dependent upon the bioavailability of GnRH from the hypothalamus, because immunization against GnRH markedly reduced LH concentrations in the long term and the LH response to secretagogues. In contrast, FSH concentrations were less affected by immunization, and there appear to be components of FSH secretion and perhaps production that are relatively independent of GnRH bioavailability.

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