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Delayed Implantation Caused by Administration of Sheep Immunogamma Globulin against LHRH in the Rat[1] (39331)

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The indispensability of the pituitary gland for the maintenance of early pregnancy in the rat was documented as early as 1933 by Pencharz and Long (1). In the same year, Selye *et al.* (2) also reported that pituitary gonadotropin plays an important role in the first half of pregnancy in the rat, since hypophysectomy before Day 11 of pregnancy terminated pregnancy. Pregnancy of rats hypophysectomized before Day 7 was maintained by gonadotropin(s) combined with prolactin (3-5) or prolactin combined with estrogen (5, 6). More recent studies using antiserum to LH have shown that antiserum to LH administered prior to Day 8 of pregnancy delayed implantation (7, 8) or caused abortion in the rat (8-11). Delayed implantation resulting from antiserum to LH was restored by administration of estrogen (12), suggesting that the antiserum which may have neutralized the activity of endogenous LH caused a reduction of estrogen secretion.

It is well established that the secretion of LH and probably FSH as well is regulated by LHRH. If the secretion of gonadotropins during early pregnancy is controlled by the hypothalamus by means of LHRH, neutralization of the activity of endogenous LHRH by a specific antiserum to LHRH would affect early pregnancy. The information on the effect of administration of anti-LHRH serum on pregnancy would shed light on the role of the hypothalamus in establishing pregnancy. The present study deals with the effect of sheep anti-LHRH gamma globulin on implantation of fertilized ova in the rat.

Methods and materials. Adult rats of Charles River, CD strain were used throughout the experiment. They were maintained in an animal quarter artificially illuminated from 0500 to 1900 hr daily. The animals were given free access to Purina Laboratory chow and water. One week after arrival, vaginal smears were checked in the female rats every morning as described by Everett *et al.* (13), and only rats exhibiting at least two consecutive 4-day cycles were selected for this study. On the afternoon of proestrus (P), one female rat was caged with two male rats which had been proven fertile. The next morning, the vagina of the female rat was inspected for the presence of spermatozoa by vaginal lavage with 0.9% saline. If spermatozoa were present, that day was designated as pregnancy Day 1.

One milliliter of sheep anti-LHRH gamma globulin (anti-LHRH-G) or normal sheep gamma globulin (NSG) was injected into the jugular vein of the rat under ether anesthesia between 0900 and 1000 hr on the day indicated in the Results.

Blood was collected from the jugular vein of some of these animals on various days during the pregnancy. All animals underwent laparotomy under Nembutal anesthesia (3.5 mg/100 g BW) on Days 8 and 14 for inspection of implantation sites. Some of the animals were allowed to survive to term.

Antiserum to LHRH was generated in two ewes by immunizing the animals with multiple injections of Glu[1]-LHRH (Dr. D. H. Coy) which was conjugated with human serum albumin (HSA). Three-and-one-half milligrams of HSA-Glu[1]-LHRH conjugate was injected into each ewe at 2-week intervals as described elsewhere (14). One of these ewes (No. 772) produced the antibody to LHRH with a singificant titer after the fifth immunization.

Anti-LHRH-G was isolated from the antiserum by ammonium sulfate by two-step precipitation procedures (15), then dis-

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solved in 0.9% saline and dialyzed against 0.9% saline for 72 hr at 4° to remove ammonium sulfate. The dialyzed gamma globulin was adjusted for volume by adding 0.9% saline to make up the original serum volume. Ten-milliliter aliquots were placed in vials, lyophilized, and stored at 4°. Shortly before the experiment, the content of each vial was dissolved in 10 ml of H_2O. Gamma globulin from normal sheep serum was similarly prepared.

Antigenic determinants of the anti-LHRH-G were determined by examining the cross reactivity with various synthetic peptides corresponding to fragments of LHRH decapeptide, as described elsewhere (16). Those peptides shown in Table I were kindly supplied by Dr. Yanaihara.

Serum samples collected from rats at various stages of pregnancy were determined for LH and prolactin by radioimmunoassay (RIA). Serum LH was determined in duplicate by RIA as described by Niswender *et al.* (17), (OO-Rat-LH RIA) using NIH-LH-S 17 as the reference standard. Serum prolactin was measured in duplicate by RIA as described by Niswender *et al.* (18) using NIAMDD-RAT-Prolactin kit. The assay data of LH and prolactin were processed by a computer program for RIA described by Duddleson *et al.* (19). Mean serum hormone levels of each group were compared with those of others by using Duncan's new multiple range test. Synthetic LHRH preparation used (TAP 023) was kindly provided by Dr. M. Fujino, Takeda Chemical Industries. 17-β Estradiol was obtained from Schering Corp. (N.J.).

Results. Characterization of sheep anti-LHRH-G. Sheep anti-LHRH-G (no. 772) bound 99% of ^{125}I-LHRH at 1:70 dilution under the conditions of radioimmunoassay for LHRH (14). The antibody-tracer binding was inhibited by unlabeled LHRH in a dose-related manner, enabling us to establish a linear standard curve using logit of B/Bo% on the ordinate and log dose on the abscissa in a range from 16 to 4096 pg/tube, where B and Bo represented bound radioactivity with and without the presence of unlabeled LHRH. The antibody-tracer binding was not affected by other hypothalamic and pituitary hormones, including TRH, somatostatin, rat LH, FSH, and prolactin, and the extracts of the rat placenta. However, the binding was inhibited to various degrees by synthetic peptides corresponding to fragments of LHRH or LHRH analogs. As shown in Table I, only peptides which contained Leu-Arg-Pro-Gly-NH_2 showed significant cross-reactivity. Therefore, the antigenic determinant at the antibody side is considered to reside in this amino acid sequence.

Effect of anti-LHRH-G on implantation. The number of implantation sites on Days 8 and 14 of pregnancy in rats injected with anti-LHRH-G or NSG are summarized in Table II. Fourteen implantation sites on the average were clearly observed on Day 8 in the rats given NSG from Days 1 through 7 (Group 1). On Day 14, a similar number of

TABLE I. CROSS-REACTIVITY OF SHEEP ANTI-LHRH GAMMA GLOBULIN (NO. 772) WITH VARIOUS SYNTHETIC PEPTIDES CORRESPONDING TO FRAGMENTS OF LHRH DECAPEPTIDE.

Peptide	Amino acid sequence	Cross-reactivity, %
LHRH	*p*Glu·His·Trp·Ser·Tyr·Gly·Leu·Arg·Pro·Gly·NH_2	100
1	*p*Glu ——————Leu·OH	0
2	*p*Glu ——————Gly·OH	0
3	*p*Glu ——Trp·OH	0
4	His ——————Leu·OH	0
5	Ser ————Leu·OH	0
6	Leu ————Gly·NH_2	30
7	Ser ——————Gly·NH_2	70
8	*p*Glu( )——————Gly·NH_2	100
9	*p*Glu( )——————Gly·NH_2	100
10	His ——————Gly·NH_2	100
11	*p*Glu ——————Gly·OH	0
12	Trp ——————Pro·OH	0
13	Trp ——————Gly·NH_2	100
14	Trp ——————Arg·NH_2	0

TABLE II. EFFECT OF ANTI-LHRH-G[a] ON IMPLANTATION IN RATS.

Group	Number of rats	Treatment	Duration of treatment	Mean implantation sites (Number of rats with implantation sites) Day 8	Day 14	Remarks
1	5	NSG[b]	Days 1-7	14 (5)	13 (5)	Normal parturition
2	8	Anti-LHRH-G	Days 1-7	0 (0)	5 (3)	Two delivered pups 7-8 days after term
3	6	NSG	Days 3-5	13 (6)	13 (6)	Normal parturition
4	12	Anti-LHRH-G	Days 3-5	0 (0)	13 (9)	Parturition delayed by 4-6 days
5	5	Anti-LHRH-G	3	13 (5)	14 (5)	Normal embryonic swellings on Day 14; normal parturition
6	6	Anti-LHRH-G	4	5 (1)	9 (4)	Parturition delayed by 3 – 4 days
7	6	Anti-LHRH-G	5	13 (6)	13 (6)	Some fetuses small on day 14; normal parturition
8	6	Anti-LHRH-G + 2 × 1 μg of LHRH/ 16% gelatine	4	13 (6)	12 (6)	Normal embryonic swellings on Day 14
9	5	Anti-LHRH-G + 1 μg of estradiol	4	10 (5)	10 (5)	Nearly normal, some small on Days 8 and 14
10	5	Anti-LHRH-G + 0.02 μg of estradiol	4	12 (4)	14 (4)	Most small on Day 14
11	6	Anti-LHRH-G + 0.01 μg of estradiol	4	12 (1)	14 (6)	Indistinguishable on Day 8, small on Day 14

[a] Sheep anti-LHRH gamma globulin.
[b] Normal sheep gamma globulin.

viable fetuses were present, and the animals delivered pups on term. When rats were injected with 1 ml of anti-LHRH-G once daily from Days 1 through 7 of pregnancy (Group 2), no implantation site was recognized on Day 8. On Day 14, three out of eight rats had viable fetuses but they were smaller than those found in NSG-treated control rats (Group 1). Two of these rats delivered pups but the parturition was delayed by 7–8 days.

Other groups were treated with anti-LHRH-G (Group 4) or NSG (Group 3) from Days 3 through 5. On Day 8, no implantation site could be distinguished in the rats treated with anti-LHRH-G, but in 9 out of 12 rats, embryonic swelling became evident on Day 14. These rats delivered pups 4–6 days later than term. The results indicate that treatment with 1 ml of anti-LHRH-G from Days 3 through 5 delayed implantation by about 5 days but did not terminate gestation.

To pinpoint the critical time at which LHRH is essential for timely implantation, 1 ml of LHRH was injected iv on Day 3, 4, or 5 of pregnancy. A clearcut inhibition of implantation on Day 8 was observed when anti-LHRH-G was injected on Day 4, but no effect was obtained when anti-LHRH-G was administered on Day 3 or 5. In the rats treated on Day 4, implantation appeared to have been delayed by 3 days, judging from the day of parturition.

Prevention of delayed implantation by LHRH or estrogen. On Day 4, the rats of group 8 received one iv dose of anti-LHRH-G and two sc doses of LHRH in 16% gelatin/0.9% saline. Implantation sites on Day 8 were nearly normal (Table II). On Day 4 of pregnancy, different doses of estradiol were also injected together with anti-LHRH-G. A dose of 0.02 μg of estradiol injected concomitantly with anti-LHRH-G only slightly restored the delayed implantation. A nearly complete nullification of the inhibition effect of anti-LHRH-G on implantation was achieved by 1 μg of estradiol. These results suggest that delayed implantation by anti-LHRH-G is caused by neutralization of endogenous LHRH and, in turn, by the decline of estrogen secretion produced by this treatment.

Plasma LH levels on Day 4 or 5 appeared

to be lower after anti-LHRH-G injection. However, since the preinjection levels were in a range close to the margin of assay sensitivity (from nondetectable to 1.9 ng/ml), quantitative measurement of the reduction of plasma LH was impossible to make. There was no significant difference in plasma prolactin levels between groups treated with NSG and those treated with anti-LHRH-G.

Discussion. The results of the present study clearly indicate that hypothalamic LHRH is indispensable on Day 4 of pregnancy in rats for the timely implantation of fertilized ova, most probably by maintaining secretion of LH and, in turn, estrogen. Our studies indicate that serum LH in castrated rats declines as early as 2 hr after iv injection of sheep anti-LHRH serum (Vilchez-Martinez *et al.*, unpublished observation) and that injection of the anti-LHRH serum to proestrous hamsters at noon blocks midafternoon elevation of serum LH (20). These findings indicate that the blockade of the action of endogenous LHRH by exogenously administered antibody occurred within a few hours after injection. The fact that anti-LHRH-G could delay implantation only when injected on Day 4 of pregnancy, not on Day 3 or 5, suggests that Day 4 of pregnancy is the critical period when the hypothalamic hormone is most needed for a successful implantation. This also suggests that the effect of anti-LHRH-G does not last longer than 1 day.

Although we could not determine serum estrogen, the restoration by estradiol of the delay in implantation induced by anti-LHRH-G indicates that secretion of estrogen which is essential for implantation was suppressed below the threshold required for nidation. Although estrogen appears to play a major role in implantation, the function of progesterone, together with estrogen, in the process of implantation on Day 4 of pregnancy cannot be excluded. Plasma progesterone levels show two peaks during pregnancy in rats: the first small peak appears on Days 3–5 and a large peak on Days 13–20, implying a physiological significance at these periods (21). Plasma progesterone levels during early pregnancy are indeed decreased by the administration of anti-LHRH-G (22).

Daily injections of anti-LHRH-G from days 1 through 7 of pregnancy not only inhibited implantation on Day 8, but also on Day 16 in some animals. In some rats, resorption of fetuses was observed at the second laparotomy. This could be due to decreased secretion of progesterone which is essential for viability of the implanted fetuses (7, 21).

Loewit and Laurence (23) and Madhwa Raj and Moudgal (24) reported that administration of anti-LH serum to rats from Days 7 through 14 of pregnancy prevented or terminated gestation. Progesterone, however, given concomitantly with the antiserum to LH, overcame the inhibitory effect of anti-LH, and the pregnancy was maintained.

The results of the present study on antisera to LHRH resemble the findings obtained by using a minimum effective dose of anti-serum to LH which caused delayed implantation, but which did not terminate gestation (12). Madhwa Raj *et al.* (12) reported that the antiserum given to rats on the morning of Day 4 of pregnancy resulted in an inhibiton of implantation on Day 8 and that an LH surge precedes an estrogen surge on Day 4 of pregnancy. On the other hand, Morishige *et al.* (25) and Linkie and Niswender (26) reported elevated serum LH levels during early pregnancy in rats, from Days 1 through 11 and Days 1 through 4, respectively, but they did not find a significant LH surge on the afternoon of Day 4 of pregnancy. In the present study we could not observe clear-cut suppression of serum LH by anti-LHRH due to the limited sensitivity of the assay. However, our findings imply that the maintenance of basal secretion of LH during early pregnancy requires LHRH. It is possible that administration of antiserum reduces serum LH below the threshold levels for implantation.

When our findings on the effect of anti-LHRH-G are compared with those of others who have examined the effect of antiserum to LH, it appears that the effect of anti-LHRH-G is less pronounced than that of anti-LH serum. LH in circulation may be abruptly and nearly completely neutralized by the excess dose of antiserum, resulting in a sudden disappearance of the hormone. On the other hand, LHRH which is mainly con-

fined to the median eminence and hypophysial portal blood may be subjected to the neutralizing action of exogenously administered anti-LHRH-G. This reduction of circulating LH by anti-LHRH may take place more gradually and less drastically than that caused by anti-LH serum, and the circulating LH would never fall to zero because of the residual function of pituitary gonadotrophs. Absence of vaginal bleeding in all of the rats given anti-LHRH-G in the present study could support this speculation.

Summary. The effect of the administration of sheep anti-LHRH gamma globulin (anti-LHRH-G) on implantation of fertilized ova was investigated in rats. Daily injections of 1 ml of anti-LHRH-G from Days 1 through 7 of pregnancy uniformly inhibited implantation of fertilized ova on Day 8, but viable sites, though considerably smaller in size than in control rats, became distinguishable on Day 14 in most rats. In some of these rats resorption of fetuses occurred, and others delivered pups 7–8 days after term. When the rats were given anti-LHRH-G from Days 3 through 5, the implantation was delayed by 5 days, but the gestation was not terminated. A single injection of 1 ml of anti-LHRH-G on Day 4 inhibited implantation on Day 8, but injection on Day 3 or 5 did not. The delayed implantation by anti-LHRH-G injected on Day 4 was nullified by concomitant administration of 2 sc injections of 1 μg of LHRH, or a single dose of 1 μg of estradiol. The data indicate that the hypothalamic LHRH is essential on Day 4 of pregnancy for timely implantation of fertilized ova, probably by maintaining LH and, consequently, estrogen secretion.

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