IMMUNOBIOLOGICAL ACTIONS OF ANTISERUM TO OVINE PROLACTIN IN MICE

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Summary. Neutralization of pituitary prolactin by the administration of antiserum to prolactin in the mouse selectively blocked the pro-oestrous stage of the oestrous cycle and inhibited ovulation in normal cyclic mice. In pseudopregnant mice, the antiserum shortened the duration of pseudopregnancy. Failure of the antiserum to affect uterine cell division, blastocyst implantation and pregnancy suggests that the secretion of progesterone by the luteal tissue appears to be more influenced by LH than by prolactin.

INTRODUCTION

In the female, it is well documented that FSH brings about the maturation of the ovarian follicles and the subsequent growth of the ovaries. On the other hand, LH has been associated with the secretion of oestrogen, with the consequent stimulation of ovulation and the conversion of the follicle into a CL (Donovan & Lockhart, 1972). Prolactin has been shown to exert a luteotrophic action on newly formed CL and a luteolytic effect on the older CL (Malven & Sawyer, 1966; Grandison & Meites, 1972). Although these three gonadotrophins are generally implicated in the control of ovarian function, the role of the latter seems to vary from species to species.

Studies in our laboratory have demonstrated that antiserum to ovine LH inhibited the endogenous LH activity in immature rats. Continuous injections of antiserum in the mouse prevented the occurrence of normal oestrous cycles, decreased the weights of the reproductive organs, inhibited blastocyst implantation and caused resorption of implanted fetuses (Munshi & Rao, 1967). A single injection of antiserum to LH given during the preimplantation stage of pregnancy impeded the production of progesterone, thereby inhibiting pregnancy as well as the decidual cell reaction in pseudopregnant mice (Munshi *et al.*, 1972). Treatment with LH antiserum did not affect implantation of the blastocysts (Munshi & Nilsson, 1973) or uterine cell division (Munshi *et al.*, 1973). Antiserum to ovine FSH, however, did not have any deleterious effect on any of the reproductive functions in the mouse (Munshi & Rao, 1968).

Prolactin is known to be secreted by the pituitary gland during pro-oestrus. Several workers have reported that concentrations of serum prolactin are high

during pro-oestrus and oestrus, and reach peak levels during the afternoon of pro-oestrus (Kwa & Verhofstad, 1967; Niswender *et al.*, 1969; Gay *et al.*, 1970; Wuttke & Meites, 1970; Neill *et al.*, 1971). Macdonald *et al.* (1971) demonstrated that prolactin prolongs the life-span of the CL. Astwood & Greep (1938) and Cerrutti & Lyons (1960) have reported that the placenta secretes prolactin. It was of interest therefore to investigate whether antiserum to prolactin would have deleterious effects on any of the reproductive functions in the mouse.

MATERIALS AND METHODS

Hormones

The ovine prolactin (NIH-P-S10) and ovine growth hormone (NIH-GH-S10) used in this study were prepared and characterized for the Endocrinology Study Section by Dr L. E. Reichert, Jr. The GH activity in NIH-P-S10 was reported to be less than 0.010 i.u./mg. The prolactin activity in NIH-GH-S10 was reported to be less than 3 i.u./mg.

Animals

The inbred Swiss-strain female mice and the rabbits used in the study were from the animal colony of the Institute. Four to five mice were caged together and maintained in an animal room at a constant temperature of 70°F and a lighting schedule of 12 hr light/12 hr dark. Rabbits weighing 2.5 kg were used for the preparation of antiserum.

Preparation of the antiserum

Antiserum to ovine prolactin was prepared in rabbits according to the method of Munshi & Rao (1965), except that the booster dose of prolactin was injected intraperitoneally. The double diffusion technique of Ouchterlony (1949) was used to characterize the antiserum. The precipitin test (Kabat & Mayer, 1961) and the haemagglutination test (Rao & Sadri, 1959) using cells treated with formalin (Raghavan *et al.*, 1973) was used for the quantification of the antibodies in the antiserum.

The analytical polyacrylamide disc gel electrophoretic procedure of Davis (1964) was employed with slight modifications. A sample of about 200 µg of protein in 10 to 30 µl of 0.05 M-NaHCO₃ buffer (pH 9.2) was placed over the spacer gel. The volume was made up to 0.1 ml with 0.5 M-sucrose and mixed thoroughly. The upper reservoir buffer was placed slowly on it to form a separate layer. Electrophoresis was carried out using tris-glycine buffer, pH 8.9. Unstained, longitudinally sliced polyacrylamide gels were embedded in 1.5% agar solution in 0.15 M-sodium chloride on a glass Petri dish, immediately following the electrophoresis. After solidification of the agar, a 2-mm slit was cut on both the sides of the gels and at a distance of 4 mm. The slits were filled to the brim with unabsorbed or absorbed antiserum to ovine prolactin. The plates were kept in aqueous saturated chambers at 4°C for 2 to 3 days.

Pooled antiserum (ASOP) absorbed free of GH contaminant was used for the biological studies.

The growth hormone (GH) contaminant in the antiserum was removed by absorption with water-insoluble immunoabsorbents of GH prepared by the method of Avrameas & Ternynck (1969) involving a 2.5% glutaraldehyde solution as a cross-linking agent. The immunoabsorbent was stored at 4°C, centrifuged before use and the supernatant was discarded. The polymerized antigen so obtained was resuspended in 50 ml of the antiserum to ovine prolactin, stirred and kept for 60 min at room temperature. The supernatant was centrifuged at 3000 rev/min for 15 min and the precipitate was discarded. The supernatant antiserum was used in the studies reported here.

Effect of pooled antiserum on ovulation

Oestrous cycles were followed by examining daily vaginal smears for 2 weeks before beginning the experiments. Mice with regular cycles were selected and five groups, each consisting of seven to eight mice, were used for the study. The first group of mice served as controls. The second group was injected subcutaneously with 0.1 ml normal rabbit serum (NRS)/day for 15 days. The third group of mice received a single subcutaneous injection of 0.2 ml ASOP on the day of dioestrus. In the fourth group, 0.2 ml ASOP was injected on the day of metoestrus and the day of dioestrus. The fifth group of mice received 0.2 ml ASOP daily for the duration of one complete oestrous cycle, and the last group received 0.2 ml ASOP for 15 days. Daily vaginal smears were taken during the entire treatment period. All mice were killed either the day after the last injection or at first oestrus. At autopsy, the oviducts of all the mice were examined for the presence of ova. Control mice, maintained for the same period of time as the experimental groups, were also killed during pro-oestrus and their oviducts were examined for the presence of ova. The ovaries and uteri were dissected free of fat and weighed to the nearest 0.1 mg on a torsion balance. The ovaries were fixed in Bouin's fixative, embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin for light microscopy.

Effect of pooled antiserum on CL function in pregnant mice

Pro-oestrous females were left overnight with fertile males and examined for vaginal plugs the next morning. The day on which a vaginal plug was found was designated Day 1 of pregnancy.

To study the effect of ASOP on uterine cell division, pregnant mice were injected on Days 1 to 3 of pregnancy. The mice in one group were killed on Day 3 and those in another group on Day 4 of pregnancy, 2 hr after a subcutaneous injection of colchicine (0.1 mg/0.1 ml saline). Adequate numbers of untreated and NRS-treated controls were also maintained. At autopsy, the uteri were fixed in Bouin's solution, embedded in paraffin wax and serial transverse sections of the middle portion of each uterine horn were cut at 5 µm and stained with haematoxylin and eosin. Mitotic counts were made in two fields located on each side of the uterine lumen of each horn according to the method of Finn & Martin (1967).

To study the effect of ASOP on pregnancy, the mice in five groups consisting of five to fifteen mated females were injected on various days of pregnancy.

Those in the first group received 0.2 ml NRS daily throughout the gestation period. The mice in the second, third, fourth and fifth groups were subcutaneously injected with 0.2 ml ASOP on Days 1 to 3, 6 to 8, 11 to 13 and 17 to 19 of pregnancy respectively. Treated mice, except those from the second and third groups, were killed on Day 19 of gestation. Mice in the second and third groups were killed on Day 12 of gestation. The numbers of implantation sites or fetuses were carefully noted at autopsy.

Effect of pooled antiserum on CL function in pseudopregnant mice

Pro-oestrous mice were mated with vasectomized males and examined the next day for a vaginal plug. The day on which a vaginal plug was observed was designated Day 1 of pseudopregnancy. Pooled antiserum was injected subcutaneously on Days 1 and 2 of pseudopregnancy while controls received NRS injections on the same days. The last day of a dioestrous vaginal smear indicated the duration of pseudopregnancy in these mice.

To study the effect of ASOP on decidual cell response, pseudopregnant mice in two groups were injected on Days 1 to 4 with 0.2 ml NRS, or ASOP, respectively. Those in a third group received a single injection of ASOP only on Day 4 of pseudopregnancy. All mice were subjected to laparotomy on Day 4 of pseudopregnancy and one horn of the uterus was traumatized by scratching with a 22-gauge needle. Mice were killed on Day 8 of pseudopregnancy and the two uterine horns were removed and weighed separately.

RESULTS

Characteristics of the prolactin antiserum

The antiserum used did not cross-react with either rabbit or sheep serum or with extracts of the pituitary, ovary, adrenal, liver, kidney, spleen, testes, uterus and placenta of the mouse as judged by the results with the Ouchterlony double-diffusion agar plates. The antiserum showed cross-reactions only with prolactin and growth hormone and not with any other ovine hormone preparation (Pl. 1, Fig. 1). Antiserum to prolactin absorbed with water-insoluble immunoabsorbent of growth hormone gave only one precipitin band with

EXPLANATION OF PLATE 1

FIG. 1. Cross-reactions of the antiserum to ovine prolactin. A, unabsorbed antiserum; 1, ovine prolactin; 2, ovine growth hormone; 3, ovine luteinizing hormone; 4, normal sheep serum.

FIG. 2. Disc gel immunodiffusion analysis of ovine growth hormone and prolactin. 1, Unstained longitudinally sliced disc electrophoresis gel column of normal sheep serum; 2 and 6, unabsorbed antiserum to ovine prolactin; 3, unstained longitudinally sliced disc electrophoresis gel column of ovine prolactin; 4, antiserum to ovine prolactin absorbed with ovine growth hormone; 5, unstained longitudinally sliced disc electrophoresis gel column of ovine growth hormone.

FIG. 3. Cross-reactions of the antiserum to ovine prolactin absorbed with ovine growth hormone. A, absorbed antiserum; 1, ovine growth hormone; 2, ovine prolactin.

FIG. 4. The relationship between the disc immunodiffusion patterns of ovine GH and ovine prolactin. A, segment of unstained gel column of ovine prolactin containing the prolactin bands; B, a segment of unstained gel column of ovine GH corresponding to the prolactin bands; C, antiserum to ovine prolactin absorbed with ovine GH.

PLATE 1

(Facing p. 414)

prolactin (Pl. 1, Fig. 3). The haemagglutination titre of the antiserum was 1:40,000 for prolactin-coated cells and as low as 1:10 for GH-coated cells.

In the quantitative precipitin test, solutions containing different aliquots of ovine prolactin (2 to 40 μg) in saline were added to a 0.05 ml absorbed antiserum to ovine prolactin. After incubation and centrifugation at 3000 rev/min, the supernatant was decanted carefully. The precipitate obtained was washed with saline and dissolved in 0.1 N-NaOH. The protein content was estimated by the method of Lowry *et al.* (1951). The results indicated that the absorbed antiserum behaved as a homogeneous system against ovine prolactin, to give a bell-shaped precipitin curve. The maximum precipitation was obtained at a concentration of 10 μg ovine prolactin (Text-fig. 1).

TEXT-FIG. 1. Quantitative precipitin test for ovine prolactin ($\blacktriangle$) and ovine growth hormone ($\times$). The quantity of antiserum to ovine prolactin in each tube was 0.05 ml.

A similar experiment was carried out with ovine GH (1 to 500 μg) and absorbed antiserum to ovine prolactin. A low degree of cross-reaction was observed between ovine GH and the antiserum (Text-fig. 1). The highest protein complex was found at a concentration of 50 μg ovine GH, a value which was three times less than that given by ovine prolactin at the concentration of 10 μg .

With polyacrylamide gel electrophoresis, ovine prolactin gave three closely situated bands towards the anode, while GH gave multiple bands of which three were intensely stained and slow moving. The fast-moving bands corresponded in their electrophoretic mobility to those given by ovine prolactin.

The disc immunodiffusion patterns of ovine GH and prolactin against unabsorbed and absorbed antisera to ovine prolactin are shown in Pl. 1, Fig. 2. Prolactin gave a broad precipitin arc against unabsorbed and absorbed antiserum to ovine prolactin, in the region of the three bands given by prolactin. Growth hormone gave two precipitin arcs, one in the region corresponding to the prolactin bands and the other a faint arc in the region corresponding to the intensely stained slow-moving bands situated towards the cathode. This additional precipitin arc was not seen on the gel column of GH and absorbed antiserum to ovine prolactin. Normal sheep serum did not show any cross-reaction with the antiserum.

The immunological relationship between the disc immunodiffusion patterns of ovine GH and prolactin against absorbed antiserum was investigated. A portion of unstained gel column of ovine prolactin containing the prolactin bands (Segment A) and that of GH in the region corresponding to prolactin bands (Segment B) were cut out, embedded in agar and allowed to cross-react with absorbed antiserum to ovine prolactin. The precipitin arc given by Segment A of ovine prolactin fused with that given by Segment B of GH (Pl. 1, Fig. 4).

Table 1. Effect of pooled antiserum on ovulation and ovarian and uterine weights of cyclic female mice

<i>Treatment group*</i> (no. of mice)	<i>No. of mice with eggs in oviducts</i>	<i>Ovary wt (mg/100 g body wt)</i>	<i>Uterine wt (mg/100 g body wt)</i>
Controls (7)	7/7	36.9 ± 5.65	801.0 ± 118.0
NRS injected for 15 days (8)	8/8	35.5 ± 6.50	745.3 ± 255.0
ASOP injected on day of dioestrus (8)	2/8	40.3 ± 5.56	1187.0 ± 318.0
ASOP injected on days of metoestrus and dioestrus (7)	0/7	34.3 ± 5.00	801.0 ± 356.0
ASOP injected during one oestrous cycle (7)	0/7	37.3 ± 6.75	591.0 ± 162.0
ASOP injected for 15 days (8)	0/8	36.3 ± 4.00	547.0 ± 165.0

* 0.2 ml pooled antiserum (ASOP) or normal rabbit serum (NRS) was injected subcutaneously at 11.00 hours.

Effect of pooled antiserum on ovulation

Control mice, untreated or injected with NRS, which were killed either on the afternoon of the day of pro-oestrus or on the morning of oestrus, had numerous ova in the oviducts. A single injection of ASOP on the day of dioestrus blocked ovulation in 6/8 mice (75%), while more than one injection of ASOP inhibited ovulation and pro-oestrus in all the animals (Table 1). Treatment with ASOP did not affect the ovarian weights of the mice, but newly formed CL were not found in any of the treated animals.

Effect of pooled antiserum on uterine cell division during early pregnancy

In mice injected with ASOP or NRS on Days 1 to 3 of pregnancy, mitotic activity was confined to the stromal cells of the uterus on Day 4.

Effect of pooled antiserum on pregnancy

As shown in Table 2, ASOP injected during the preimplantation period inhibited implantation in only 33% of the animals, but the number of implantation sites/animal was reduced. Mice injected with ASOP during the postimplantation period (Days 6 to 8) had normal numbers of implantation sites. When ASOP was injected just after placental formation (Days 11 to 13), it inhibited pregnancy in 47% of the animals with a 50% reduction in the number of young per animals. When ASOP was injected just before implantation, it had no effect.

Effect of pooled antiserum on the duration of pseudopregnancy

The ASOP injected on the first 2 days of pseudopregnancy shortened ($P < 0.01$) its duration to 10.0 ± 0.6 (S.D.) days (six mice) compared with that of controls (12.0 ± 1.4 days, seven mice).

Table 2. Effect of pooled antiserum on pregnancy in mice

<i>Treatment groups*</i>	<i>No. of mice with implantations</i>	<i>No. of implantations/mouse</i>
Controls	8/8	7.5
ASOP injected on Days 1 to 3	10/15	5.0
ASOP injected on Days 6 to 8	5/5	7.0
ASOP injected on Days 11 to 13	5/11	3.8
ASOP injected on Days 17 to 19	5/5	7.2

* 0.2 ml pooled antiserum (ASOP) was injected subcutaneously at 11.00 hours.

Effect of pooled antiserum on decidual cell response

The results showed that the weight of the traumatized uterine horn in the control mice increased threefold compared with that of the untouched uterine horn (635.4 ± 332.0 (S.D.) mg versus 243.9 ± 52.3 mg). When ASOP was injected only on Day 4 of pseudopregnancy or on Days 1 to 3, it failed to affect decidua formation and the weight of the traumatized uterine horn was similar to that of the controls (643.6 ± 326.0 versus 206.0 ± 17.1 mg, four mice, and 684.5 ± 345.0 versus 183.1 ± 33.6 mg, four mice).

DISCUSSION

Immunological studies with ovine prolactin have indicated that the hormone gives a single precipitin line against its homologous antiserum (Levy & Sampliner, 1962; Hayashida, 1962; Trenkle *et al.*, 1963; Larson & Assa, 1963; Sundaram & Sonenberg, 1969). The antiserum prepared by us also gave a single precipitin line against ovine prolactin. When the antiserum was allowed to cross-react with ovine GH, however, a slight 'lingering reactivity' was observed, perhaps due to the contamination of GH in NIH prolactin.

The results of the various immunological techniques tested indicate that the absorbed antiserum to ovine prolactin possessed a high degree of specificity.

The results presented here indicate that neutralization of endogenous prolactin, in normal cyclic female mice, caused a complete block of pro-oestrus. This effect is accompanied by inhibition of ovulation and the absence of newly formed CL in the ovaries of treated mice. Serum and pituitary prolactin levels have been reported to be very high during pro-oestrus (Niswender *et al.*, 1969; Gay *et al.*, 1970) and, in our study, the antiserum was able to neutralize this increase.

Although Niswender *et al.* (1969) have reported two peaks in serum prolactin concentration, one during placental formation and the other during parturition, the antiserum failed to cause complete resorption of implanted fetuses and did not delay or prevent parturition in our study (Table 2).

Hiroi (1971) and Macdonald *et al.* (1971) have reported ovine prolactin to be biologically active in the mouse and the rat as it prolongs the life-span of the CL in cyclic or pseudopregnant animals. Our results are in agreement with these observations since antiserum to prolactin shortened the duration of pseudopregnancy. Finn & Martin (1967) have observed that large numbers of the epithelial cells of the uterine lumen and glands but very few of the stromal cells undergo mitoses on the 3rd day of pregnancy in mice. On the 4th day, however, the situation is reversed. This change in the pattern of cell division is considered to be due to the secretion of progesterone (Martin & Finn, 1968; Finn & Martin, 1969), a factor which plays an important role in the preparation of the uterus for implantation (Finn *et al.*, 1969). The pooled antiserum thus failed to affect secretion of progesterone during early pregnancy. In contrast to our observations with antiserum to ovine LH (Munshi *et al.*, 1972), the inability of the antiserum to affect early pregnancy in mice suggests that the luteal tissue is more readily influenced by LH than by prolactin.

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