Effect of LHRH immunoneutralization on follicular development, the LH surge and luteal function in the stump-tailed macaque monkey (*Macaca arctoides*)

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Summary. A dose of 100 µl of a potent ovine LHRH gamma globulin inhibited ovulation in the cyclic rat when administered at 12:00 h on the day of pro-oestrus. A dose of 10 ml of the preparation was administered i.v. to female stump-tailed macaques to achieve circulating antibody titres 3–4-fold higher than in the rat. In an ovariectomized macaque, this caused a marked fall in serum concentrations of LH to less than 10% of pretreatment values and also a significant, though less pronounced, fall in FSH. Six monkeys were treated with the LHRH gamma globulin during the mid-late follicular phase of the cycle. In 2 monkeys in which serum oestradiol concentrations were <100 pg/ml at the time of antibody administration, the rising oestradiol levels were abruptly suppressed and the normal mid-cycle LH surge failed to occur. Serum concentrations of LH and FSH declined to low levels for 8–10 days after which time normal follicular development occurred. In the remaining 4 monkeys in which follicular development was more advanced as indicated by serum oestradiol concentrations of >100 pg/ml, the antibodies induced either a transient decline or had no effect on the rising serum concentration of oestradiol. An LH/FSH surge followed by a rise in serum progesterone occurred in these macaques. When the antibodies were administered to a further 6 macaques, which had also been treated with oestradiol benzoate during the early follicular phase to induce an LH surge, the neutralization of LHRH again failed to block the surge even when the dose of antibody was increased to 20 ml.

The results show that LHRH antibodies were unable to block the LH surge in the macaque. They contrast with results obtained with LHRH immunoneutralization in the sheep, rat, hamster, mouse and bird and suggest that the ability of oestrogen to induce an LH surge by acting directly on the LHRH-primed anterior pituitary gland is more dominant in the primate.

Introduction

The relative importance of hypothalamic LHRH and the pituitary gonadotroph as sites of the positive feedback action of oestradiol to bring about the mid-cycle gonadotrophin surge in the primate has still to be established. Studies in which LHRH has been neutralized by injection of antibodies a short time before the preovulatory LH surge have prevented the surge and ovulation in the rat (Fraser & Gunn, 1973; Koch, Chobsieng, Zor, Fridkin & Lindner, 1973; Arimura, Debeljuk & Schally, 1974), mouse (Laing, Gosden & Fraser, 1984), hamster (de la Cruz, Arimura, de la Cruz & Schally, 1976), sheep (Fraser & McNeilly, 1982) and chicken (Fraser & Sharp, 1978). In these species therefore the association between LHRH release and the LH surge is established.

However, in the rhesus monkey withdrawal of LHRH by cutting the pituitary stalk does not

prevent the oestrogen-induced LH surge (Ferlin, Rosenblatt, Carmel, Antunes & Vande Wiele, 1979). Also, in ovariectomized rhesus monkeys with hypothalamic lesions to prevent endogenous LHRH secretion, restoration of normal serum gonadotrophin concentrations by exogenous LHRH results in the ability of oestrogen to induce an LH surge even when pulses of LHRH are stopped 48 h before oestrogen administration (Wildt, Hausler, Hutchinson, Marshall & Knobil, 1981). Furthermore, injection of LHRH antibodies failed to prevent the oestrogen-induced LH surge in ovariectomized rhesus monkeys (McCormack, Plant, Hess & Knobil, 1977). These results suggested that, in the monkey, the LH surge could be brought about by oestrogen acting directly on the anterior pituitary and that LHRH need only be present to prime the gonadotroph.

In view of our findings, and those of others, that injection of LHRH antibodies could block the LH surge in the chicken, rat, hamster, mouse and sheep (Fraser, McNeilly & Popkin, 1984, review) we decided to utilize this approach to investigate the role of LHRH in the LH surge in intact stump-tailed macaque monkeys which have menstrual cycles similar to those of women.

Materials and Methods

Preparation of LHRH gamma globulin. A potent LHRH antiserum raised in a ewe (No. 94) by immunization against a human serum albumin (HSA)–LHRH conjugate was used. Specificity was directed towards the C-terminal end of LHRH, but there was no cross-reaction with other peptide hormones (Ellis, Desjardins & Fraser, 1983). A pool of 1.1 litres antiserum was used to prepare a concentrated gamma globulin fraction with unwanted proteins removed. This was achieved by first centrifuging the antiserum at 10 000 *g* for 90 min to remove particulate material, and isolating the gamma globulin fraction by sodium sulphate precipitation (Heide & Schwick, 1978). After dialysis for 2 days against 5 changes of 3 litres 0.9% (w/v) sodium chloride at 4°C, the preparation was dialysed against sucrose for 3–4 h to reduce the fluid volume to 25% of the initial whole serum volume. The preparation was then centrifuged at 2000 *g* for 30 min at 4°C, the precipitate discarded and the supernatant stored at –40°C until used for passive immunization. LHRH antibody titre, expressed as the highest dilution binding 33% ¹²⁵I-labelled LHRH, was 1:70 000 for the neat serum and 1:160 000 for the gamma globulin preparation. A gamma globulin fraction from serum taken from a ewe immunized against HSA was prepared in a similar manner.

The biological activity of the LHRH gamma globulin was tested in cyclic female Sprague–Dawley rats housed as described previously (Popkin & Fraser, 1983) and exhibiting two 4-day oestrous cycles before study. Three groups of 6 rats were lightly anaesthetized with ether at 12:00 h on the day of pro-oestrus and injected into the tail vein with 100 µl gamma globulin to HSA (controls) or 50 or 100 µl gamma globulin to LHRH. At 17:00 h on the day of pro-oestrus a blood sample was taken from the tail vein under ether anaesthesia; serum was collected and stored at –20°C for radioimmunoassay of LH and measurement of LHRH antibody titre. The rats were killed at 09:00 h on the morning of oestrus and the number of ova shed was counted by examining the oviducts under a dissecting microscope (Fraser & Gunn, 1973).

All control rats ovulated, shedding 12–16 ova. The dose of 50 µl LHRH gamma globulin blocked ovulation in 2 of 6 rats while 100 µl was effective in all 6 rats. In control rats at 17:00 h on the day of pro-oestrus, serum concentrations of LH were elevated at 347 ± 136 ng/ml (mean $\pm$ s.e.m.) while in rats injected with 50 µl or 100 µl LHRH gamma globulin LH levels were 43 ± 5 and 40 ± 4 ng/ml, respectively, significantly lower than in control rats ($P < 0.05$, Student's *t* test). On a volume for weight basis the equivalent dose in the stump-tailed macaque would be 4 ml, but in view of the protracted events surrounding the LH surge in this species it was decided to administer a dose of 10 ml antibody.

Effect of anti-LHRH in an ovariectomized monkey. In a preliminary study to test the efficacy of the LHRH gamma globulin in the macaque, a monkey ovariectomized 1 year previously and with elevated serum concentrations of LH and FSH was injected with 10 ml of the LHRH gamma

globulin. Blood samples were collected from the femoral vein daily for 5 days, before administration of the antibody which was infused into the femoral vein via a 3-way tap over a 2-min period immediately after a blood sample had been collected. Blood samples were collected at regular intervals thereafter for a further 4 weeks.

Effect of anti-LHRH during the normal cycle. Twelve adult female stumptailed macaques with regular ovulatory menstrual cycles, as judged from serum profiles of oestradiol and progesterone and menstrual records over several years before this study, were selected. The animals were housed in two rooms at a temperature of 24–26°C open to daylight but also lit artificially between 07:00–19:00 h. They were fed a primate diet (Old World Monkey diet, B.P. Nutrition, Witham, U.K.) daily and given fresh fruit three times per week, with water available *ad libitum*. Daily blood samples were collected from the femoral vein during the cycle before treatment, the treatment cycle, and for the post-treatment cycle. The animals were used to this procedure, and it caused no apparent stress. An additional blood sample was collected at 8 h after antibody administration. The length of the follicular phase during the pretreatment cycle ranged from 10 to 15 days followed by a

Fig. 1 Effect of an i.v. injection of an LHRH gamma globulin on serum concentrations of (a) LH and (b) FSH in an ovariectomized stumptailed monkey: (c) shows titre of LHRH antibody obtained in the serum.

Fig. 2. Serum concentrations of LH, FSH, oestradiol and progesterone during a pretreatment control cycle and during the following cycle of a monkey (No. 70) after an i.v. injection of LHRH gamma globulin during the mid-follicular phase. Bottom panel shows titre of LHRH antibodies in the circulation. Bars show menstrual bleeding.

luteal phase lasting 14–16 days. On Day 10 of the cycle as judged from the first day of menstruation, 3 animals were infused with 10 ml HSA gamma globulin and 9 were infused with LHRH gamma globulin. For purposes of expressing results, Day 1 of the cycle was calculated from the first day of the fall of luteal progesterone to 0.3 ng/ml. In some animals, this preceded detection of menstruation by 2–3 days.

Effect of LHRH on the oestrogen-induced LH surge. Six monkeys with regular menstrual cycles were given an i.m. injection of 50 μ g oestradiol benzoate/kg (Intervet, Organon Laboratories Ltd, Morden, Surrey) in arachis oil on Days 1–5 of a control cycle, and all exhibited an LH surge beginning 36–48 h later. After 2 or 3 control cycles the same monkeys were again treated with 50 μ g oestradiol benzoate/kg at the same stage of the cycle, but this time were also given an infusion of LHRH gamma globulin 24 h later ($N = 2$) or at the same time as oestrogen administration ($N = 4$). Four of the monkeys received 10 ml LHRH gamma globulin, while 2 of the latter group received 20 ml. Blood samples were collected at 0, 12, 24, 36, 48, 60, 72, 84, 96 and 108 h after oestrogen injection.

Radioimmunoassays. Oestradiol and LH were measured by radioimmunoassays as described by Fraser, Laird & Blakeley (1980) and progesterone as described by Fraser, Baird, McRae, Nestor & Vickery (1985). Three heterologous radioimmunoassays for FSH were compared for measurement

of pools of serum from an intact male, an intact female and an ovariectomized stump-tailed macaque. All results were measured against the rhesus monkey pituitary gonadotrophin preparation LER 1909-2. Antibodies M91, M94 and NIAMDKD hFSH 5 were all raised to human FSH in rabbits. Tracers tested were 125 I-labelled rat FSH (r FSH-1-5; NIAMDKD), human FSH (CPDS-13; Dr S. S. Lynch) and ovine FSH (oFSH-181; Professor M. Jutisz). Use of antiserum hFSH5 gave a sensitivity of 120 ng/tube and maximal displacement of tracer to only 30%. Best results were obtained using M94 in combination with ovine FSH tracer as described previously for sheep (McNeilly, McNeilly, Walton & Cunningham, 1976). Sensitivity was less than 40 ng/tube and maximal displacement to less than 5% tracer with 2500 ng/tube. At an initial dilution of 1:2000, 12–25% counts were specifically bound. Parallel displacement was achieved with all 3 pools of serum. Samples were assayed at 200 μ l in duplicate. Inter- and intra-assay coefficients of variation were 8 and 10% respectively.

LH bioassay. Serum LH concentrations were measured by a modification of the mouse Leydig cell bioassay (Steiner & Bremner, 1981), originally described by Van Damme, Robertson & Diczfalussy (1974) and Dufau, Hodgen, Goodman & Catt (1977). All plasma samples were assayed in triplicate at volumes of 1 or 20 μ l. The assay sensitivity was 0.15 μ g/ml. The intra- and interassay coefficients of variation were 7.9% and 12.1% respectively.

Fig. 3. Serum concentrations of LH, FSH, oestradiol and progesterone during a pretreatment control cycle and during the following cycle of a monkey (No. 58) after an i.v. injection of LHRH gamma globulin during the late follicular phase. Bars show menstrual bleeding.

Fig. 4. Serum concentrations of LH, FSH, oestradiol and progesterone during a pretreatment control cycle and during the following cycle of a monkey (No. 71) after an i.v. injection of LHRH gamma globulin on the day after the preovulatory gonadotrophin surge. Bars show menstrual bleeding.

Results

Effect of anti-LHRH in an ovariectomized monkey

At 6 h after administration of LHRH gamma globulin the circulating titre of LHRH antibody in the ovariectomized monkey, as expressed as the highest dilution binding 33% ¹²⁵I-labelled LHRH, was 1:9000 falling to 1:6400 by 24 h and, by the 3rd day, half of the antibodies had disappeared from the blood (Fig. 1). Serum concentrations of LH fell rapidly after antibody administration while FSH values fell more slowly. By 14 days, LHRH antibody titre had fallen to 1:900 and this was followed by a gradual return to elevated serum concentrations of LH and FSH.

Effects of anti-LHRH during the follicular phase

Administration of HSA gamma globulin to 3 control monkeys was without effect on follicular development and timing of the LH surge. During the pre-treatment cycle, serum concentrations of oestradiol-17 β reached a peak of 150–350 pg/ml in the follicular phase which was followed by an LH surge either on the same day or on the following day (Figs 2, 3, 4). Of the 9 monkeys treated with LHRH gamma globulin, 2 were in the mid-follicular phase as judged by serum concentrations of <100 pg oestradiol/ml, 4 were in the late follicular phase having oestradiol levels >100 pg/ml at the time of antibody administration, while the 3 remaining monkeys had had an LH surge 24 h previously.

In the 2 monkeys treated during the mid-follicular phase, the rising levels of oestradiol were rapidly reduced to values around the detection limit of the assay and the expected mid-cycle surge of LH failed to occur (Fig. 2). In the absence of a progesterone rise, a premature menstruation occurred. Serum concentrations of LH and FSH remained suppressed for 8–10 days after which time they rose to induce follicular development leading to a normal ovulatory cycle. In the 4 monkeys in which follicular development was more advanced, individual serum oestradiol concentrations at time of antibody administration being 115, 146, 203 and 232 pg/ml, the LHRH antibodies induced either a transient decline in serum oestradiol concentrations or had no effect on the rising oestradiol (Fig. 3). An LH/FSH surge of normal amplitude and duration occurred in these

Fig. 5. Effect in monkeys of oestradiol benzoate (50 µg/kg) on serum concentrations of immunoreactive and bioactive LH and on FSH on Days 1–5 of the cycle (a) and the effect of an i.v. injection of 10 ml (N = 2) or 20 ml (N = 2) of LHRH gamma globulin given at the same time as oestradiol benzoate to the same animals during a subsequent cycle (b). Values are means $\pm$ s.e.m.

animals. The duration of the subsequent elevation in serum concentrations of progesterone was normal, although in one animal was clearly of lower magnitude than normal while in the remainder there was a tendency towards low values during the early luteal phase.

Effects of anti-LHRH on luteal function

Of the 3 monkeys in which the LHRH gamma globulin was injected on the day after the LH peak, one had an apparently normal serum progesterone profile while 2 had only a small, short-lived rise in progesterone lasting 5–8 days and premature menstruation (Fig. 4). After 10 days, serum LH and FSH concentrations began to rise and this was followed by a cycle with normal hormone profiles.

Effect of anti-LHRH on the oestrogen-induced LH surge

The first 2 monkeys treated with LHRH antibodies 24 h after administration of oestradiol benzoate both had rises in LH and FSH similar to pretreatment control test (data not shown). The remaining 4 monkeys treated with 10 or 20 ml LHRH gamma globulin at the time of oestradiol administration in an effort to achieve longer suppression of LHRH also showed LH and FSH responses of similar amplitude to those of the control test (Fig. 5). In these monkeys levels of immunoreactive LH failed to show any evidence of negative feedback after oestrogen and rose sooner than during the control test. In contrast, LH bioactivity showed a clear fall in LH 12 h after oestradiol benzoate in the test with LHRH-antibody ($P < 0.001$, paired t test). However, in agreement with the radioimmunoassay, during the test with LHRH-antibody treatment the LH surge had commenced by 36 h in all animals compared to 48 h for the control test.

Comparison of LHRH antibody titres in rats and monkeys

Serum from rats injected with 100 μ l LHRH gamma globulin at 12:00 h on the day of pro-oestrus contained LHRH antibody titres of between 1:2000 and 1:3500 at 17:00 h of pro-oestrus and between 1:1600 and 1:2000 at 09:00 h of oestrus. As expected, sera from all monkeys treated with the antibody had titres 3–4 times these amounts over the equivalent time period (Figs 1–4). In addition the serial dilutions set up to assess antibody titres showed that when the LH surge was occurring in the LHRH antibody-treated monkeys a 1:1000 dilution of the monkey's serum could still bind > 90% of the LHRH tracer.

Discussion

The failure of the LHRH gamma globulin to block the preovulatory LH surge when administered during the late follicular phase, or the LH surge induced by oestradiol benzoate, was surprising in view of our previous studies in which we had used similar ovine LHRH antisera to prevent the LH surge or ovulation in the chicken (Fraser & Sharp, 1978), mouse (Laing *et al.*, 1984), rat (present study), hamster (H. M. Fraser, unpublished observations) and ewe (Fraser & McNeilly, 1982; McNeilly, Fraser & Baird, 1984). One obvious explanation for the results was that the antibodies were ineffective in neutralizing the endogenous LHRH. However, lesser titres of the same gamma globulin fraction were sufficient to block the LH surge and ovulation in the rat. The antibody also markedly suppressed the serum concentrations of LH and FSH in an ovariectomized monkey and blocked the cycle when administered during the mid-follicular phase in 2 monkeys. This antiserum had been previously shown to block LH pulses (Ellis *et al.*, 1983) and LHRH receptor rises in castrated rats (Clayton, Popkin & Fraser, 1982), suppress serum concentrations of LH and testosterone and LHRH receptor numbers in intact male and female rats (Clayton *et al.*, 1982;

Fraser, Sharpe, Lincoln & Harmer, 1982; Popkin & Fraser, 1983), suppress LHRH receptor numbers in male mice (Naik *et al.*, 1985) and suppress serum testosterone concentrations in male monkeys (Cameron *et al.*, 1985).

There is no doubt that chronic manipulation of LHRH does prevent the oestrogen-induced LH surge in the stump-tailed macaque as shown by active immunization against LHRH (Fraser, 1983) or after the opposite approach of chronic LHRH agonist administration (Fraser, 1981). Both these approaches induced chronic changes in stimulation of the gonadotroph. The present results, suggesting that oestrogen can act directly on the LHRH-primed pituitary to induce an LH surge when LHRH was neutralized for a discrete period, would confirm and extend several other observations in the ovariectomized rhesus monkey. For example, McCormack *et al.* (1977) failed to block the oestrogen-induced LH surge by using a rabbit antiserum to LHRH. Ferin *et al.* (1979) obtained oestrogen-induced LH surges after pituitary stalk section and placement of a Silastic barrier between the hypothalamus and pituitary. Wildt *et al.* (1981) were able to elicit an LH surge in ovariectomized rhesus monkeys in which secretion of endogenous LHRH had been blocked by hypothalamic lesions, provided the pituitaries had been primed with exogenous LHRH pulses up to 48 h before oestrogen administration. Stopping pulses at this time still resulted in an LH surge. These and other (Knobil, 1980) findings have given rise to the concept that, in the primate, oestrogen can act directly on the primed pituitary gland to induce an LH surge, providing it with a 'fail-safe' mechanism not found in other species.

However, these findings contradict those of Norman, Gliessman, Lindstrom, Hill & Spies (1982) who found that ovulatory cycles could not be restored by LHRH pulses in monkeys with hypothalamic lesions and with a Teflon barrier placed between the hypothalamus and pituitary. The conclusion that hypothalamic LHRH is necessary during the LH surge is supported by the prevention of the surge in ovariectomized rhesus monkeys by an LHRH antagonist (Asch *et al.*, 1983).

The ability of the antibodies in the present study to block follicular development in the mid-, but not the late, follicular phase may be due to increased sensitivity and prolonged responsiveness of the mature follicle to the gonadotrophin stimulation before LHRH antibody administration or to low levels of gonadotrophins produced after the antibody. While it was not possible to measure discrete changes in gonadotrophins in the macaques, studies in sheep show that LHRH antibodies cause an immediate cessation of LH pulses, but low levels of LH may be maintained (Fraser & McNeilly, 1983; McNeilly *et al.*, 1984).

The possibility that the macaque pituitary is more sensitive than that of other species to low levels of LHRH, remaining active despite the administration of large amounts of a potent antiserum, must also be considered. The resulting circulating titre achieved in the monkeys was higher than that which blocked ovulation in the rat and higher than that obtained by us after successful immunoneutralization of LHRH in the ram (Lincoln & Fraser, 1979) and in the ewe (McNeilly *et al.*, 1984). In addition, in the present study we showed that, during the time of the LH surge, a dilution of 1:1000 of the monkey's serum was still able to bind more than 90% of the LHRH tracer *in vitro*. In view of the high binding of LHRH achieved in these monkeys and the effectiveness of the antiserum in other species we consider that further attempts to increase circulating antibodies would be unjustified and impracticable. We are unable to offer an explanation for the apparent early onset of the LH surge in the monkeys given LHRH antibodies at the time of oestradiol benzoate administration.

The fact that 2 of the 3 monkeys injected with antibodies to LHRH on the day after the LH surge had only a small and short-lived rise in serum progesterone would suggest that the primate corpus luteum is gonadotrophin-dependent. This would agree with recent studies showing declining progesterone concentrations in blood after administration of a potent LHRH antagonist in the stump-tailed macaque (Fraser *et al.*, 1985). Also, in rhesus monkeys with hypothalamic lesions in which ovulation had been induced with exogenous LHRH and then this stimulation stopped during the luteal phase (Hutchinson & Zeleznik, 1984), serum concentration of progesterone fell

prematurely. The fact that serum progesterone concentrations were not markedly altered in macaques receiving the LHRH antibody before the LH surge may be because the rapidly declining antibody concentrations allow low levels of LH to be released, sufficient to maintain luteal function. This could also explain why luteal function was apparently unaffected by hypophysectomy or by a less potent LHRH antagonist (Asch, Abou-Samra, Braunstein & Pauerstein, 1982; Balmaceda, Borghi, Coy, Schally & Asch, 1983).

In conclusion, the results indicate that the primate differs from other species in that oestrogen can induce an LH surge despite immunoneutralization of LHRH. This would agree with studies using several other approaches to investigate the site of action of oestrogen in bringing about the LH surge in the rhesus monkey. However, as equally convincing investigations have yielded opposite results we feel our conclusions must be tempered by considering other possibilities, a difference in sensitivity to small amounts of LHRH remaining after immunoneutralization being the most obvious.

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