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Effect of continuous infusion of a low dose of GnRH antagonist on serum LH and testosterone concentrations, spermatogenesis and semen quality in the rhesus monkey (*Macaca mulatta*)

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Summary. Treatment of 4 adult male rhesus monkeys for 8–12 months with 100–400 μg of a GnRH antagonist/day by means of using osmotic minipumps led to suppressed serum concentrations of LH and testosterone followed by various degrees of recovery toward pretreatment values. The serum LH response to a challenge of native GnRH was reduced by 30–75% during antagonist treatment. The serum testosterone response to GnRH was exaggerated above the response in the pretreatment period, suggesting hypersensitivity of the testis to gonadotrophin. Antagonist administration under these conditions did not alter body weight or abolish ejaculatory response. Antagonist infusion caused a 96% decrease in sperm counts. Spermatozoa recovered during the final month of antagonist treatment showed a reduced ability to penetrate denuded hamster ova. Testicular biopsies performed at the end of antagonist treatment revealed persistent spermatogenesis. However, the cellularity of the seminiferous tubules was decreased below that of pretreatment biopsies. The results of this study suggest that the amount of testosterone needed to maintain normal spermatogenesis is greater than that needed to maintain electroejaculatory response in monkeys.

Introduction

The development of gonadotrophin-releasing hormone (GnRH) antagonists has led to a number of attempts to use these analogues to induce azoospermia. However, administration of large doses has led to undesirable side effects. Daily injection or continuous infusion of milligram doses of GnRH antagonists for 9 weeks induced azoospermia in male monkeys, but was associated with a significant loss of body weight (Weinbauer *et al.*, 1984; Akhtar *et al.*, 1985). Subcutaneous administration of large doses of the antagonist, Ac-(2)D-NAL^1-pF-D-Phe^2-D-Trp^3-D-Arg^6-GnRH, caused facial oedema and oedema of the extremities in rats and reduced spontaneous activity in monkeys (Schmidt *et al.*, 1984). These results suggested that it might be impossible to attain reliable azoospermia without significant side effects, thus reducing the possibility of using GnRH antagonists for contraception.

However, the provision of low levels of GnRH agonist by continuous infusion is an effective way of suppressing LH and testosterone secretion and the response to a GnRH challenge (Akhtar *et al.*, 1983; Mann *et al.*, 1984). In this study, we examine the effect of continuous infusion of a GnRH antagonist via osmotic minipump on basal LH and testosterone secretion and the response to an i.v. bolus of GnRH in male rhesus monkeys. In addition, we investigated the effect of low-dose continuous infusion of GnRH antagonist treatment on sperm count and quality.

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Materials and Methods

Four adult male rhesus monkeys were infused continuously with a GnRH antagonist (Ac-(2)D-NAL[1]-pF-D-Phe[2]-D-Trp[3]-D-Arg[6]-GnRH) for 36 or 50 weeks using a s.c. implanted (intrascapular area) osmotic minipump (Alza Corporation, Palo Alto, CA; model 2 ml-4). Two monkeys (A and B) were treated with 100 μg/day for 10 weeks, then 200 μg/day for 20 weeks and finally 400 μg/day for an additional 20 weeks. The other 2 animals (C and D) were treated, with the same antagonist, with 200 μg/day for 16 weeks and then 400 μg/day for 20 weeks. New pumps were implanted every 4 weeks. Animals were maintained in a temperature- (23°C) and light- (12 h light:12 h dark) controlled room throughout the study. Blood samples (4 ml) were taken by venepuncture from the saphenous vein of restrained conscious animals during the pretreatment period (3–5 samples), at 3 and 7 days of treatment and thereafer at weekly intervals. Serum samples were stored frozen at −20°C until assayed for LH and testosterone.

At monthly intervals throughout the study, all animals were injected with an i.v. bolus of 25 μg synthetic GnRH (United States Biochemical Corporation, Cleveland, OH) to test the serum LH and testosterone response. Blood samples were collected 15 min before administration, just before administration and at 15, 30, 60 and 120 min after GnRH injection. Serum samples were stored at −20°C until LH and testosterone were measured.

Serum concentrations of LH were determined using the mouse interstitial cell bioassay (Van Damme *et al.*, 1974) with the NIADDK rhesus monkey pituitary gonadotrophin standard (LER 1909-2) as the reference preparation. The intra- and interassay coefficients of variation were 14·1 and 14·7% respectively. The minimal detectable dose for LH was 0·2 μg/ml. Serum concentrations of testosterone were determined by radioimmunoassay as previously described (Perachio *et al.*, 1977). The intra- and interassay coefficients of variation for testosterone were 5·1 and 6·8%. The minimal level of detection for the testosterone assay was 0·5 ng/ml.

Testicular measurements were made at monthly intervals with Lange calipers with a compression pressure of 15 g as previously described (Mann *et al.*, 1985). The formula used for testicular volume was $4/3\ a^2b$ (a = short axis; b = long axis). Testicular needle biopsies were performed on all animals during the pretreatment period, at the end of antagonist treatment and at 16 weeks of recovery. Tissue was processed for routine histological examination as previously described (Mann *et al.*, 1985). Biopsy samples were embedded in paraffin wax, cut at 4 μm and stained with haematoxylin and eosin or with Masson's trichrome for connective tissue. The mean ± s.e.m. of the number of seminiferous tubules collected from biopsies was 34·6 ± 4·7 (range 10–47).

Animals were subjected to rectal probe ejaculation at approximately 4-week intervals as reported previously (Gould *et al.*, 1978) to determine sperm counts, percentage live spermatozoa and percentage motile spermatozoa. Sperm samples from all treated monkeys, and from 4 untreated, age-matched animals housed with the treated monkeys, were tested for their ability to penetrate denuded hamster oocytes during the last month of antagonist treatment and again 16 and 22 weeks after the end of antagonist treatment. The hamster ova penetration test was performed essentially as described by Yanagimachi *et al.* (1976). Hamster oocytes were recovered from superovulated hamsters (25 i.u. PMSG i.p. followed by 50 i.u. hCG i.p. 3 days later). The cumulus cells were removed by incubating in 1% hyaluronidase for 15 min and the zonae were dissolved by transferring the oocytes to a 0·1% trypsin solution in Dulbecco's medium for 10 min. After dissolution of the zonae pellucidae, the denuded oocytes were transferred to Ham's F10 medium containing 10% human cord serum. After washing, 10 oocytes were cultured in 0·5 ml culture medium in each well of an 8-well culture dish (Lab Tek, Inc., Naperville, IL).

Semen samples were washed twice in culture medium, concentrated by centrifugation and further washed by allowing motile spermatozoa to 'swim up' into Ham's culture medium which had been overlaid on the sperm pellet. The 'swim-up' procedure took 30–60 min. Washed spermatozoa were added to the denuded oocytes at a ratio of 25 000 motile spermatozoa/oocyte, and 10–30 oocytes were used per assay. Incubations were performed at 37°C. At 18 h the oocytes were compressed under a cover slip and stained with aceto-orcein or lacmoid. The assay was scored according to the percentage of oocytes penetrated per well. After penetration, the sperm head undergoes swelling and decondensation, and can be identified by using phase-contrast microscopy.

The statistical significance of all results, except those from the hamster ova penetration assay, was assessed by analysis of variance and the least significant difference for multiple comparisons as needed (Keppel, 1973). The results from the hamster ova penetration assay were tested for statistical significance by χ^2 test.

Results

The effects of GnRH antagonist infusion on serum LH and testosterone for Monkeys A and B are shown in Fig. 1. Administration of 100 μg antagonist/day induced an immediate fall in serum LH values in Monkey A, and these remained suppressed throughout the period of treatment. In this animal, serum testosterone concentrations decreased initially, but then recovered to pretreatment values before declining again when the dose of antagonist was increased to 200 μg/day, and remaining below pretreatment values until treatment was terminated. Monkey B was not as responsive to the antagonist; there was no consistent suppression of serum LH or testosterone concentrations even after the dose was increased to 400 μg/day. Instead, there were alternate periods of suppression and

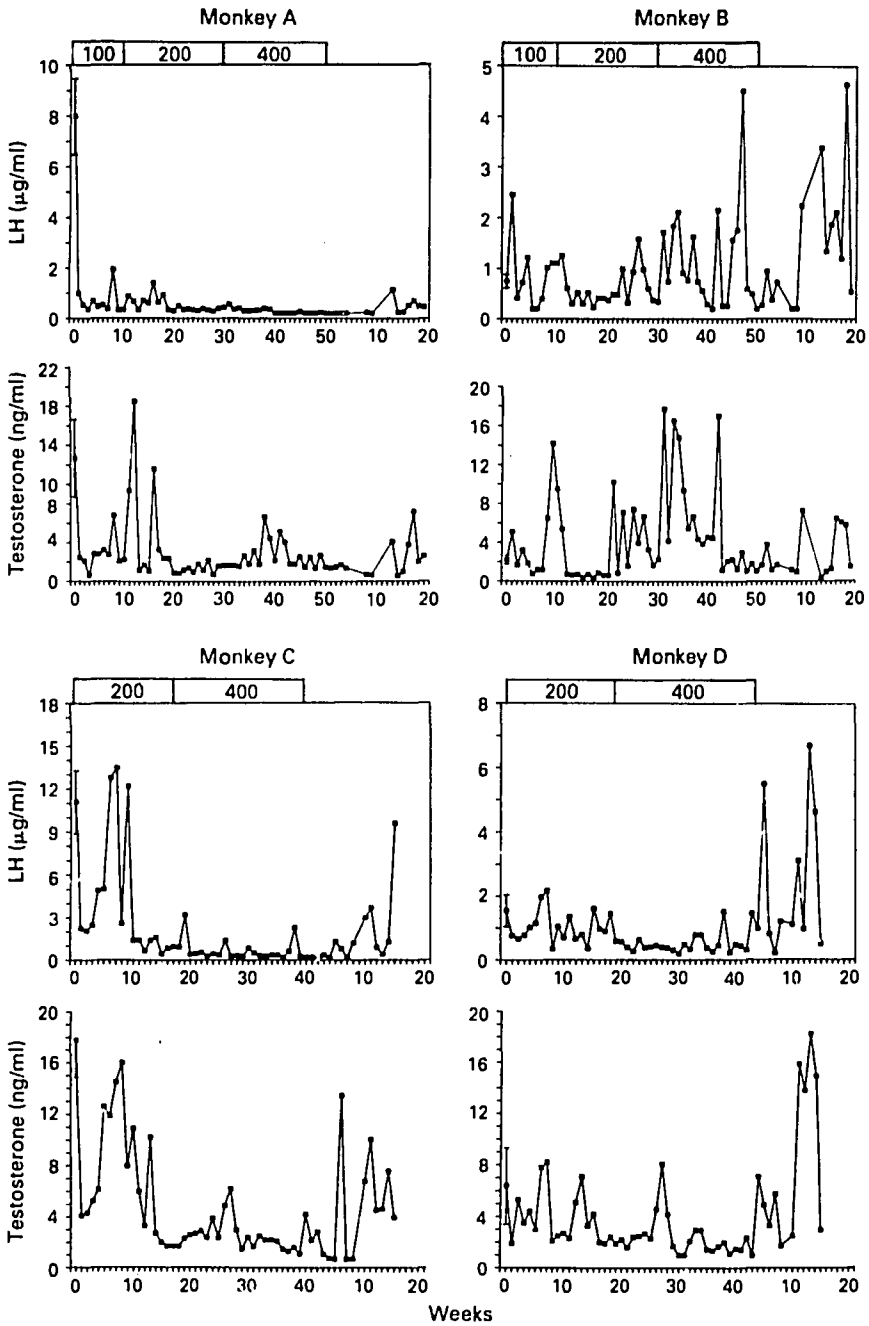

Fig. 1. Influence of continuous GnRH antagonist infusion on serum LH and testosterone concentrations in Monkeys A, B, C and D. The doses of antagonist administered (µg/day) and the length of administration are shown at the top of the figure. The total treatment period was 50 weeks for monkeys A and B and 36 weeks for Monkeys C and D.

recovery. The lack of suppression of LH and testosterone by 100 µg antagonist/day in the Monkeys A and B led to deletion of this treatment in Monkeys C and D and treatment began with 200 µg/day (Fig. 1). Serum LH and testosterone concentrations fell initially, showed some recovery and then declined again to subnormal values.

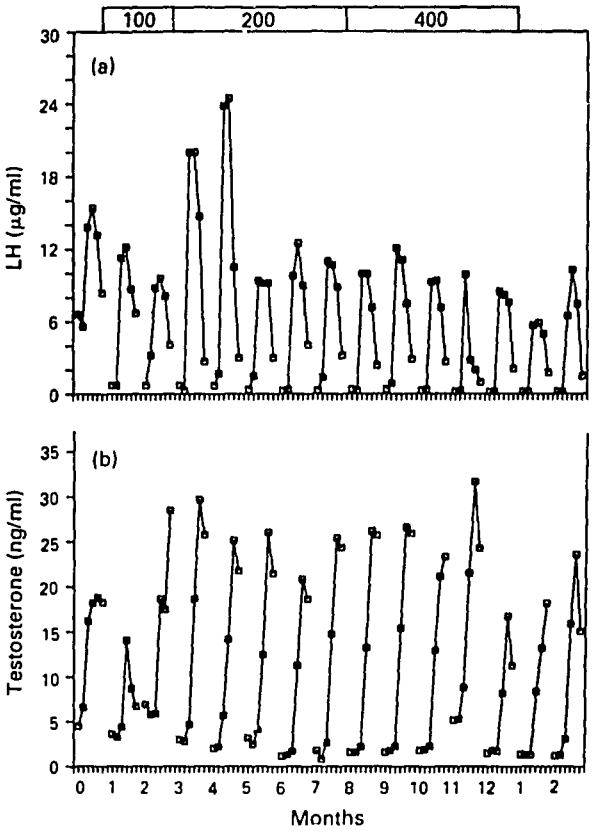

Fig. 2. Typical serum LH (a) and testosterone (b) response to 25 µg GnRH in Monkey A before (0 months), at monthly intervals during 12 months of antagonist administration and at 1 and 2 months of the post-treatment period. Blood samples were taken at −15, 0, +15, +30, +60 and +120 min of GnRH infusion. The doses of antagonist administered (µg/day) and the length of administration are shown at the top of the figure. The total treatment period was 50 weeks.

Figure 2 shows a typical serum LH and testosterone response to a challenge with 25 µg GnRH in Monkey A. The peak serum LH response to GnRH was decreased initially by antagonist treatment, recovered to values that exceeded the response in the pretreatment period, then fell again to values that were about 40% of the pretreatment response when the dose of antagonist was increased. The serum testosterone response to GnRH was elevated above the pretreatment response throughout antagonist administration, suggesting hypersensitivity of the testis to LH. The other 3 animals showed a similar response to GnRH administration.

Mean (±s.e.m.) sperm count decreased ($P < 0{\cdot}05$) over the course of GnRH antagonist infusion (Fig. 3a), from $7{\cdot}1 \pm 2{\cdot}4 \times 10^8$/ml during the pretreatment period to $0{\cdot}29 \times 10^8$/ml during the last month of antagonist infusion. The percentages of motile and live spermatozoa in ejaculates (Fig. 3b) also decreased during the treatment period, but not statistically significantly so.

During the final month of antagonist treatment, spermatozoa from treated monkeys showed a reduced ability ($P < 0{\cdot}005$) to penetrate denuded hamster ova (Fig. 4). Spermatozoa from control animals (age matched; housed under similar conditions to the treated monkeys) showed a mean ± s.e.m. penetration rate of $27{\cdot}7 \pm 4{\cdot}2$% compared with $5{\cdot}5 \pm 2{\cdot}0$% for spermatozoa from antagonist-treated monkeys. When the test was repeated 16 weeks after the end of antagonist treatment, spermatozoa from 3 of 4 treated monkeys showed a normal penetration rate. The sample from the

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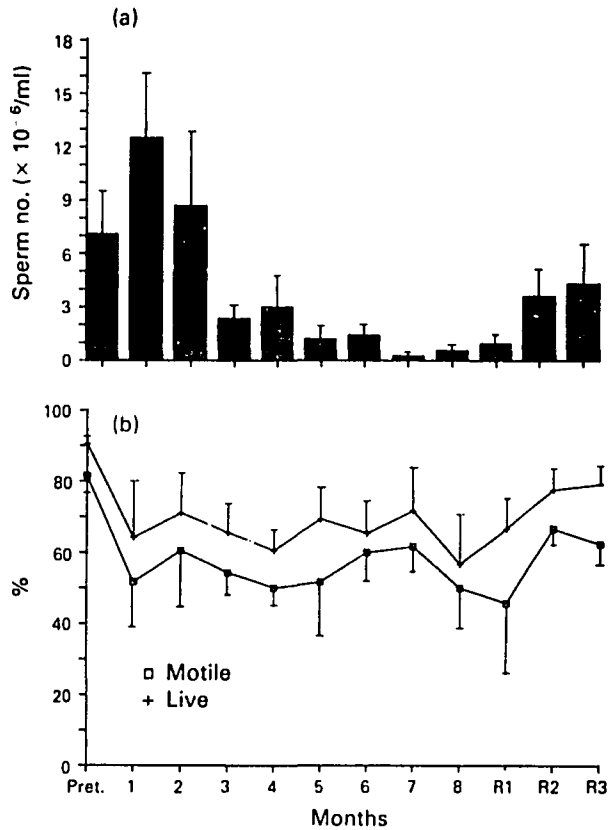

Fig. 3. Effect of continuous GnRH antagonist administration on mean ± s.e.m. sperm count (a) and % motile and % live spermatozoa (b) in the ejaculates of the 4 monkeys. Pret. = pretreatment; R = recovery period.

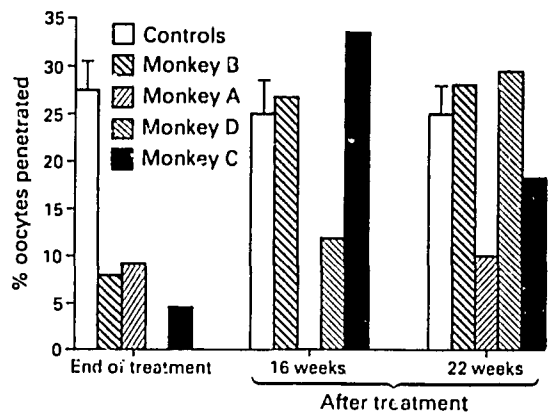

Fig. 4. Effect of GnRH antagonist administration on the mean ± s.e.m. percentage penetration of hamster ova by spermatozoa from untreated control monkeys and from individual animals treated by a GnRH antagonist. The test was run on all monkeys during the last month of treatment, and at 16 and 22 weeks after treatment.

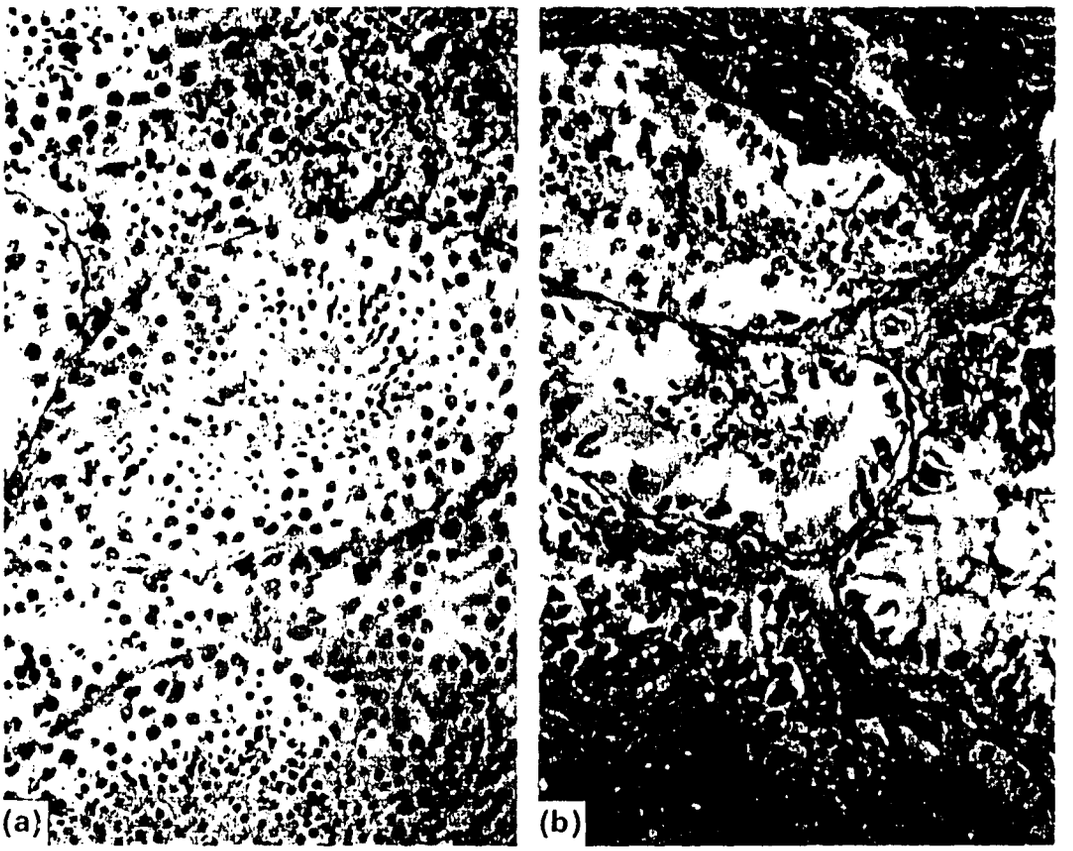

Fig. 5. Effect of GnRH antagonist treatment on testicular histology in Monkey A: (a) before treatment and (b) at the end of treatment. × 200.

4th animal, Monkey A, did not penetrate hamster ova, but when the test was repeated 7 weeks (22 weeks after treatment) later, the penetration rate had increased to 10%. By 26 weeks after the end of treatment, the penetration rate of spermatozoa from Monkey A was 37·5%. This monkey also showed a slower return to normal basal concentrations of serum LH and testosterone after the end of antagonist treatment (see Fig. 1).

In the antagonist-treated monkeys, active spermatogenesis persisted, as indicated by the presence of germ cells representative of all stages of spermatogenesis, including spermatids (Fig. 5), but though present, spermatids were less numerous than in pretreatment biopsies. There was no thickening of the lamina propria, or other evidence of intertubular fibrosis. No areas of calcification were noted.

The testicular volumes of the 4 animals treated with the GnRH antagonist are shown in Fig. 6. Testicular volumes declined more than 45% in the 4 monkeys during antagonist treatment. In Monkey B, in which it was not possible to maintain suppressed concentrations of serum LH and testosterone (see Fig. 1), testicular volume increased between 5 and 8 months of antagonist treatment before declining again.

The mean ± s.e.m. of pretreatment body weights (8·9 ± 0·5 kg) did not differ from body weights (9·4 ± 0·6 kg) at the end of treatment. None of the 4 monkeys lost weight over the course of antagonist administration. We did not observe any effect of antagonist treatment on spontaneous activity nor was there any noticeable peripheral or facial oedema in the monkeys.

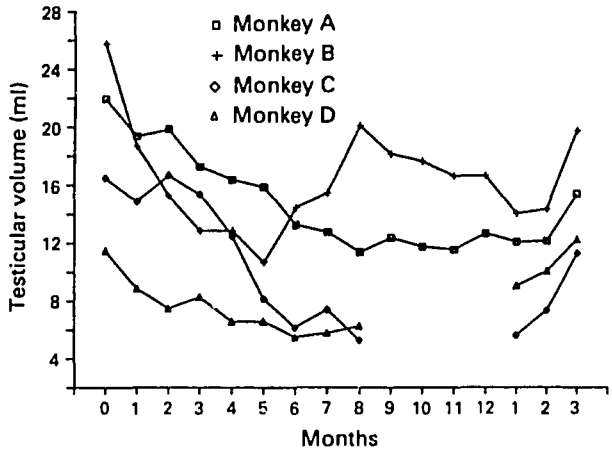

Fig. 6. Effect of GnRH antagonist treatment on testicular volume in the 4 monkeys (Monkeys A and B were treated with antagonist for 12 months, and Monkeys C and D for 8 months). The final 3 measurements were made on each animal at 1, 2, and 3 months after treatment.

Discussion

The inhibitory effect of GnRH antagonists on gonadal function apparently relates to their ability to bind with high affinity to pituitary GnRH receptors, preventing binding by native GnRH (Clayton *et al.*, 1982). There is no evidence that antagonists alter the turnover rate of the GnRH receptor (Bex & Corbin, 1984). In the present study, serum LH and testosterone concentrations showed an immediate fall with the onset of antagonist treatment, but then recovered toward pretreatment values after 8–10 weeks of antagonist administration (100 μg/day). This recovery may result from compensation by the hypothalamus and/or pituitary to the reduced levels of LH, and increased sensitivity of the pituitary to GnRH. Our data support this mechanism. During the periods that LH and testosterone showed recovery during antagonist treatment, the serum LH response to GnRH was supernormal. Furthermore, the serum testosterone response to GnRH was elevated above the pretreatment response throughout antagonist administration, suggesting increased sensitivity of the gonads to LH in the presence of reduced serum concentrations of gonadotrophin.

Two other explanations are possible for the recovery from antagonist treatment. It is possible that antibodies against GnRH were generated, leading to a reduced suppression. This is unlikely from this small decapeptide, although it cannot be excluded because of its highly modified nature relative to native GnRH. A second possibility is that the GnRH antagonist loses its potency during the 4 weeks it is present in the minipump. This explanation can be excluded by the fact that serum LH and testosterone concentrations were not suppressed again when pumps were replaced, but only by increased levels of the GnRH antagonist.

While there was suppression of LH and testosterone in some animals with continuous low doses of the antagonist, the suppression was not complete and serum LH and testosterone values tended to increase after a period of treatment. These results clearly suggest that the mechanism for inhibition by GnRH antagonists is very different from the continuous administration of small amounts of a GnRH agonist. Agonist treatment leads to initial stimulation of LH followed by down regulation of the GnRH receptor and marked suppression of LH concentrations (Heber & Swerdloff, 1981). On the other hand, the antagonists suppress LH by a receptor occupancy mechanism (Clayton *et al.*, 1982).

At the doses of antagonist used in this study, we did not encounter the side effects reported by previous workers (Schmidt *et al.*, 1984; Weinbauer *et al.*, 1984; Akhtar *et al.*, 1985)). Two different GnRH antagonists (*N*-Ac-D-p-Cl-Phe^{1,2}-D-Trp^{3}-D-Arg^{6}-D-Ala^{10}-GnRH and *N*-Ac-D-Nal(2)^{1}-D-

p-Cl-Phe^2-D-Trp^3-D-Arg(Et$_2$)^6-D-Ala^{10}-GnRH) caused a significant loss of weight when administered in daily doses sufficient to induce azoospermia (Weinbauer *et al.*, 1984; Akhtar *et al.*, 1985). The antagonist used in this study was the same analogue which caused oedema in rats and reduced spontaneous activity in monkeys (Schmidt *et al.*, 1984). We did not observe any apparent side effects in our animals, possibly because the doses used were only 5–10% of those previously used and were given at constant rates.

Testicular biopsies performed at the end of antagonist treatment (8–12 months) in the 4 monkeys revealed active spermatogenesis, but decreased numbers of spermatogenic cells in the seminiferous tubules. There was no evidence of tubular calcification or fibrosis. A final needle biopsy performed at 16 weeks of recovery showed that the testicular morphology was fully recovered from antagonist treatment. The effects of the antagonist on testicular histology in monkeys differ markedly from those obtained using a potent GnRH agonist (Mann *et al.*, 1985). The agonist caused a total blockade of spermatogenesis within 20 weeks of treatment with diffuse atrophy of the seminiferous tubules. Tubules contained only a small number of spermatogonia and no evident spermatids. Part of the difference in the effects of the two analogues on spermatogenesis may relate to the degree of suppression of testosterone concentrations. In the antagonist-treated animals, serum testosterone values were suppressed below pretreatment levels but were above the values in agonist-treated monkeys.

The serum testosterone concentration needed to maintain normal spermatogenesis may be greater than that necessary to maintain ejaculatory response. Antagonist administration greatly reduced sperm counts in the present study, but an apparently normal electroejaculatory response was maintained. This suggests that a dose of GnRH antagonist may be found that would induce azoospermia or functional azoospermia (oligospermia with reduced potential fertilizing capacity of remaining spermatozoa) without the undesirable side effects reported by previous investigators (Schmidt *et al.*, 1984; Weinbauer *et al.*, 1984; Akhtar *et al.*, 1985) or loss of ejaculatory response.

Our results show that continuous infusion of 100–400 μg/day of a GnRH antagonist caused oligospermia, although azoospermia was not achieved. These results contrast with those of previous studies in which doses 5–10-fold higher did induce azoospermia (Weinbauer *et al.*, 1984; Akhtar *et al.*, 1985). Additional studies with a higher dose of GnRH antagonist administered by continuous infusion will be necessary to determine whether a dose of GnRH antagonist can be identified which gives azoospermia without undesirable side effects or loss of ejaculatory response. Our study suggests that it may be possible to achieve reliable contraception without attaining azoospermia. Spermatozoa from GnRH antagonist-treated animals penetrated only 6% of denuded hamster ova versus 27% by spermatozoa from untreated animals, suggesting that GnRH antagonist-treated male monkeys have a reduced fertilizing potential.

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References

Akhtar, F.B., Marshall, G.R., Wickings, E.J. & Nieschlag, E. (1983) Reversible induction of azoospermia in rhesus monkeys by constant infusion of a gonadotropin-releasing hormone agonist using osmotic minipumps. *J. clin. Endocr. Metab.* **56**, 534–540.

Akhtar, F.B., Weinbauer, G.F. & Nieschlag, E. (1985) Acute and chronic effects of a gonadotropin-releasing hormone antagonist on pituitary and testicular function in monkeys. *J. Endocr.* **104**, 345–354.

Bex, F.J. & Corbin, A. (1984) LHRH and analogs: Repro-

ductive pharmacology and contraceptive and therapeutic utility. In *Frontiers in Neuroendocrinology*, vol. 8, pp. 85–151. Eds L. Martini & W. F. Ganong Raven Press, New York.

Clayton, R.N., Channabasaviah, K., Stewart, J.M. & Catt, K.J. (1982) Hypothalamic regulation of pituitary gonadotropin-releasing hormone receptors: effects of hypothalamic lesions and a gonadotropin-releasing hormone antagonist. *Endocrinology* **110,** 1108–1115.

Gould, K.G., Warner, H. & Martin, D.E. (1978) Rectal probe electroejaculation of primates. *J. med. Primatol.* **7,** 213–221.

Heber, D. & Swerdloff, R.S. (1981) Gonadotropin-releasing hormone analog and testosterone synergistically inhibit spermatogenesis. *Endocrinology* **108,** 2019–2021.

Keppel, G. (1973) *Design and Analysis: A Researcher's Handbook*. Prentice-Hall, Inc., Englewood Cliffs.

Mann, D.R., Gould, K.G. & Collins, D.C. (1984) Influence of continuous gonadotropin-releasing hormone (GnRH) agonist treatment on luteinizing hormone and testosterone secretion, the response to GnRH, and the testicular response to human chorionic gonadotropin in male rhesus monkeys. *J. clin. Endocr. Metab.* **58,** 262–267.

Mann, D.R., Smith, M.M., Gould, K.G. & Collins, D.C. (1985) Effect of a gonadotropin-releasing hormone agonist on luteinizing hormone and testosterone secretion and testicular histology in male rhesus monkeys. *Fert. Steril.* **43,** 115–121.

Perachio, A.A., Alexander, M., Marr, L. & Collins, D.C. (1977) Diurnal variations of serum testosterone levels in intact and gonadectomized male and female rhesus monkeys. *Steroids* **29,** 21–33.

Schmidt, F., Sundaram, K., Thau, R.B. & Bardin, C.W. (1984) Ac-D-NAL(2)[1], 4FD-Phe[2], D-Trp[3], D-Arg[6]-LHRH, A potent antagonist of LHRH, produces transient edema and behavioral changes in rats. *Contraception* **29,** 283–289.

Van Damme, M.-P., Robertson, D.M. & Diczfalusy, E. (1974) An improved *in vitro* bioassay method for measuring luteinizing hormone (LH) activity using mouse Leydig cell preparations. *Acta endocr., Copenh.* **77,** 655–671.

Weinbauer, G.F., Surmann, F.J., Akhtar, F.B., Shah, G.V., Vickery, B.H. & Nieschlag, E. (1984) Reversible inhibition of testicular function by a gonadotropin hormone-releasing hormone antagonist in monkeys (Macaca fascicularis). *Fert. Steril.* **42,** 906–914.

Yanagimachi, R., Yanagimachi, H. & Rogers, B.J. (1976) The use of zona-free animal ova as a test system for the assessment of the fertilizing capacity of human spermatozoa. *Biol. Reprod.* **15,** 471–476.

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