Sustained Pharmacological Activities in Rats Following Single and Repeated Administration of Once-a-Month Injectable Microspheres of Leuprolide Acetate[1]

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Once-a-month injectable microspheres of leuprolide acetate prepared with copoly(DL-lactic/glycolic acid) using an in-water drying method were assessed for duration of the analogue release and pharmacological effects in rats after a single or repeated injection. The periodic challenge test revealed that a single injection of the microspheres caused a dramatic and persistent suppression of the ability of the pituitary–gonadal system to secrete gonadotropin and testosterone for over 5 weeks. The complete recovery of these functions was observed 10 weeks after the injection. The repeated injection of the microspheres at intervals of 2 or 4 weeks achieved persistent suppression of steroidogenesis after an initial transient flare-up and beneficially avoided the "acute-on-chronic response." This depot formulation is expected to assure patient compliance and produce stronger therapeutic effects than the daily solution.

KEY WORDS: leuprolide (leuprorelin); once-a-month injectable microspheres; gonadotropin suppression; steroidogenesis inhibition; repeated administration.

INTRODUCTION

Leuprolide [leuprorelin; D-Leu^6-(des-Gly^{10}-NH$_2$)-LH-RH ethylamide] acetate, a highly active agonistic analogue of luteinizing hormone-releasing hormone (LH-RH), potently inhibits pituitary gonadotropin secretion and suppresses testicular and ovarian steroidogenesis ("chemical castration") when administered chronically in therapeutic doses. These inhibitory effects are further used in the treatment of hormone-dependent tumors (3–5) and endometriosis (6). For these applications, everyday s.c. injections over a long time period are inconvenient. To improve patient compliance and achieve more persistent efficacy, we developed once-a-month injectable microspheres of the analogue using a biocompatible and biodegradable polymer, copoly(DL-lactic/glycolic) acid (PLGA), utilizing a novel in-water drying method (7–9). This depot formulation released the analogue at a pseudo-zero-order rate over a 1-month period, and the resultant inhibition of serum testosterone and growth of the genital organs in male rats and dogs prompted us to study its effects against hormone-dependent cancers (10,11) and in an experimental rat endometriosis model (12). Sanders *et al.* (13) and Redding *et al.* (14) also developed similar sustained-release injections of the LH-RH analogues with PLGA using a phase-separation method and demonstrated well-sustained pharmacological effects in animals.

In the present study, to assess the duration of drug release from the microspheres produced by our in-water drying method, the sustained pharmacological responses as judged by the periodic challenge test after a single injection and the suppression of steroidogenesis after repeated administration at 2- or 4-week intervals were investigated in rats.

MATERIALS AND METHODS

Animals and Materials

Male Sprague–Dawley rats were purchased from Clea Japan, Inc. (Tokyo). Leuprolide acetate was synthesized in a research laboratory of our company. PLGA was purchased from Wako Pure Chemical Ind., Ltd. (Tokyo), PLGA (75.0/25.0)-14,400 for Lots 341, 342, and 343 of the microspheres, PLGA (75.7/24.3)-13,900 for Lot M06, and PLGA (76.7/23.3)-12,100 for Lot M07. The numbers in parentheses represent the lactic/glycolic molar ratio, followed by the weight-average molecular weight.

Preparation of Microspheres

The microspheres of leuprolide acetate were prepared by the method as previously described (8,9). In brief, 500 mg of the drug and 80 mg of gelatin were dissolved in 0.5 ml of distilled water at about 60°C. PLGA (4 g) dissolved in 5 ml of dichloromethane was mixed with the above solution and agitated vigorously with a homogenizer. This w/o emulsion was added to 1250 ml of 0.25% polyvinyl alcohol solution cooled to about 18°C under stirring with a turbine-shaped mixer. The resulting (w/o)/w emulsion was stirred gently for 3 hr to remove the organic solvent to obtain the microspheres. The microspheres were washed twice with water and lyophilized. The surface and cross section of the microspheres were observed under a scanning electron microscope (JSM-T-300, JEOL Ltd., Tokyo).

Drug Release *in Vivo*

Three lots of leuprolide microspheres (Lots 341, 342, 343), which were prepared in a scale-up study, were injected s.c. into the rat (6 weeks of age) at a dose of 0.9 mg of the analogue. The microspheres were excised periodically to determine the amount of drug remaining in each microspheres at the injection site (12).

Radioimmunoassay of Hormones

Serum LH and FSH (follicle stimulating hormone) were measured using RIA kits kindly supplied by Dr. A. F. Parlow (Harbor—UCLA Medical Center, Torrance, CA) (12). Values were expressed in terms of the standard rat LH

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(NIADDK-rLH RP-1) and FSH (NIADDK-rFSH RP-1), respectively. Testosterone was measured in duplicate by RIA kits from Green Cross, Co. (Osaka, Japan), using the single-antibody and dextran-charcoal method (10).

Pharmacological Responses After Injection of the Microspheres

To determine the LH and FSH serum levels, three lots of the microspheres (Lots 341, 342, 343) were injected s.c. into the rat (6 weeks of age) at a dose of 0.9 mg as the analogue, corresponding to a dose of 100 μg/kg/day of the analogue for 30 days. Blood was collected periodically from the abdominal aorta under ether anesthesia. Serum was stored below −40°C until the day of assay.

To assess the duration of inhibition of gonadotropin release and steroidogenesis, the leuprolide acetate solution (100 μg/kg) was injected s.c. at various times after the microspheres (Lot M06) had been injected s.c. [seven groups of five rats each (10 weeks of age) at a dose of 1.35 mg drug, corresponding to a dose of 100 μg/kg/day]. Blood was collected from the tail vein periodically after the analogue solution was injected to determine serum LH, FSH, and testosterone levels.

For the 2-week interval treatment, the microspheres (Lot M07) were chronically administered s.c. to five rats (10 weeks of age) at an initial loading dose of 1.35 mg drug and four succeeding maintenance doses of 0.675 mg. In another five rats, the microspheres (Lot M07) were chronically injected at 4-week intervals at a dose of 1.35 mg drug. Blood was collected from the tail vein periodically after each injection, and the serum testosterone was determined.

RESULTS AND DISCUSSION

The microspheres of leuprolide acetate prepared by the in-water drying method were spherical particles with a mean diameter of 20–30 μm and many fine pores on the surface (Figs. 1A–C). They are easily injectable using a 23-gauge needle after dispersed in the vehicle for injection. The cross-section micrograph (Fig. 1D) revealed that the microspheres were composed of a solid monolithic matrix with many narrow channels.

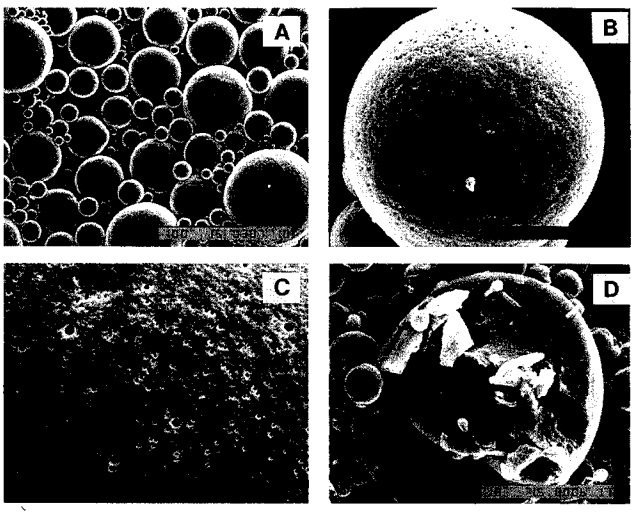

Fig. 1. Scanning electron micrographs of the surface and cross section of leuprolide microspheres. The numbers (10 and 100) at the bottom right in the photographs represent the scale in μm.

The drug trapping ratio in three lots of microspheres in a scale-up study was 95.9, 94.8, and 95.9% for Lots 341, 342, and 343, respectively. Moreover, these microspheres showed fairly similar release profiles (Fig. 2). Although the initial burst, 24.3% of the dose, increased in comparison with the small-scale preparations (12), the succeeding release progressed at an approximately zero-order rate, 2.7% of the dose/day, for 4 weeks. As shown in Fig. 3, an injection of the microspheres from any of these three lots produced the same as or lower serum levels of LH and FSH than the nonstimulated control levels in the adult rats [LH—23.9 (14–36) ng/ml, n = 8 (15), and 38–45 ng/ml, n = 25 (16); FSH—216–226 ng/ml, n = 8 (17), and 249–422 ng/ml, n = 25 (16)] for over 6 weeks after a transient initial elevation, "initial flare-up."

In general, it is difficult to encapsulate water-soluble compounds at a high rate and achieve continuous release from the microspheres (9,18). However, our microspheres of leuprolide acetate, a highly water-soluble compound, yielded a high and reproducible trapping ratio and continuous release for over 4 weeks using a biodegradable polymer with a novel preparation method. Our successful development of microspheres in which such a water-soluble compound is retained in a hydrated swelled polar polymer and is released from the matrix with the dissolution of the eroded oligomer might be explained (i) by the strong interaction between the cationized drug and the anionized polymer like that in an ion-exchange resin and (ii) by the formation of a rigid matrix structure likely resulting from a rearrangement of polymer molecules surrounding the drug cores.

Figure 4 shows the typical LH, FSH, and testosterone responses following injection of the analogue solution in untreated control rats and in rats which had been injected with microspheres 2, 4, and 6 weeks before. The serum gonadotropin levels measured before the challenge were suppressed for 5 to 6 weeks in rats which received an injection of the microspheres. The testosterone levels were depressed for 4 weeks at one-fourth to one-seventh of those in untreated normal controls [2.36 (0.33–6.50) ng/ml, n = 55 (10); 2.4 (0.9–4.2) ng/ml, n = 8 (15); 2.7–3.0 ng/ml, n = 8 (17); 3.1–4.1

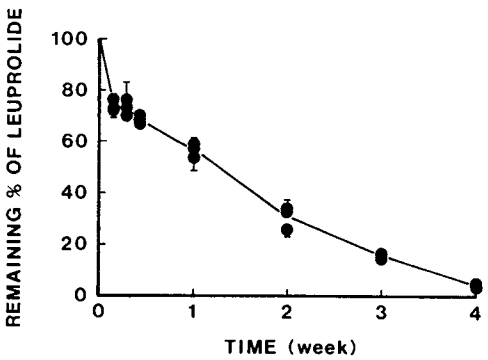

Fig. 2. Percentage of the dose of leuprolide remaining at the injection site in rats after s.c. injection using three lots of the microspheres (Lots 341, 342, 343). Each point represents the mean ± SE of five rats.

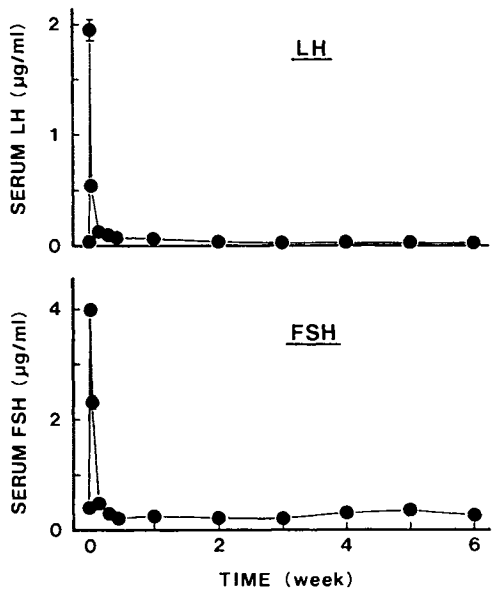

Fig. 3. Serum LH and FSH levels in rats after s.c. injection of leuprolide microspheres. Each point represents the mean ± SE of three lots of the microspheres each given to five rats. The dose was 0.9 mg/rat (6 weeks of age) of leuprolide acetate.

ng/ml, $n = 25$ (16)]. As the serum hormone levels are subject to circadian rhythm and external stimulation, these serum levels need to be compared with the normal levels in a large number of control rats. The increment AUC of LH, FSH, and testosterone serum levels for 8 hr after challenge (Fig. 5) clearly revealed the responsiveness of the pituitary–gonadal system. The release response in these organs in the treated rats was suppressed for 4 to 5 weeks following a single injection of the microspheres. After 6 weeks, a minimal gonadotropin release response reappeared, whereas the testosterone response was now readily measurable but still significantly inhibited ($P < 0.01$). Ten weeks after injection of the microspheres, complete recovery of both of these responses is observed, indicating that the "chemical castration" elicited by the chronic treatment is reversible. After daily injection of leuprolide acetate solution to female rats, the pituitary response to the challenge test recovered to 80% of control in 3 days after the last injection (19). Hence, effective drug release from the microspheres persists over 6 weeks.

The inhibition of steroidogenesis in rats after repeated injections of the microspheres at 2- or 4-week intervals is shown in Fig. 6. Drastic and consistent suppression was elicited by both of the treatments following an initial flare-up. No flare-up responses were detected after any of the subsequent maintenance dose injections of the microspheres. This depot formulation has been found to provide much stronger therapeutic effects in an experimental rat endometriosis model than the daily injection of the drug solution (12). During daily chronic treatment with the drug solution, a small testosterone release response soon after injection, "acute-on-chronic response," has been observed. Using this depot

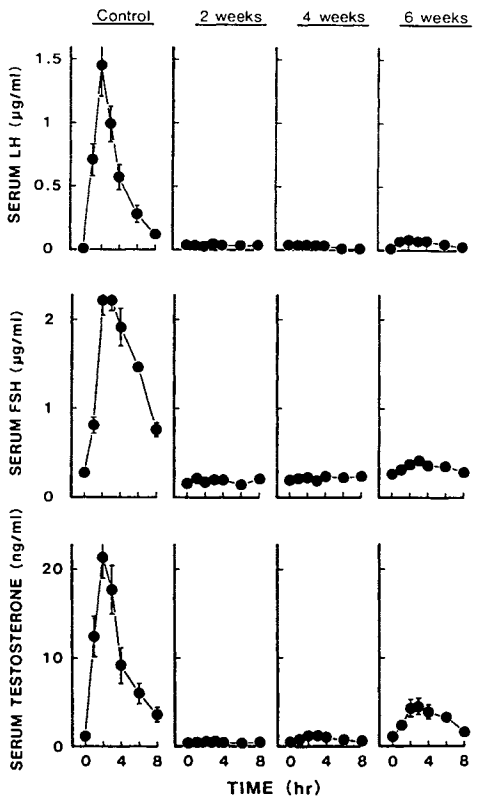

Fig. 4. Serum LH, FSH, and testosterone levels in rats following challenge using the leuprolide solution 2, 4, and 6 weeks after s.c. injection of the microspheres. Each point represents the mean ± SE of five rats. The doses of leuprolide acetate were 100 μg/kg in the solution and 1.35 mg/rat (10 weeks of age) in the microspheres.

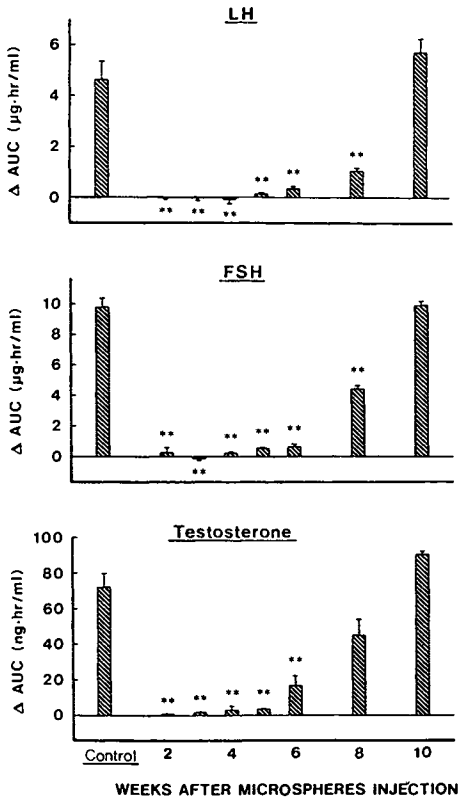

Fig. 5. Increment of AUC of serum LH, FSH, and testosterone levels for 8 hr in rats following challenge with the leuprolide solution at different times after s.c. injection of the microspheres. Each point represents the mean ± SE of five rats. The doses were the same as those indicated in Fig. 4. (**) Significantly different from nontreated control by Student's t test ($P < 0.01$).

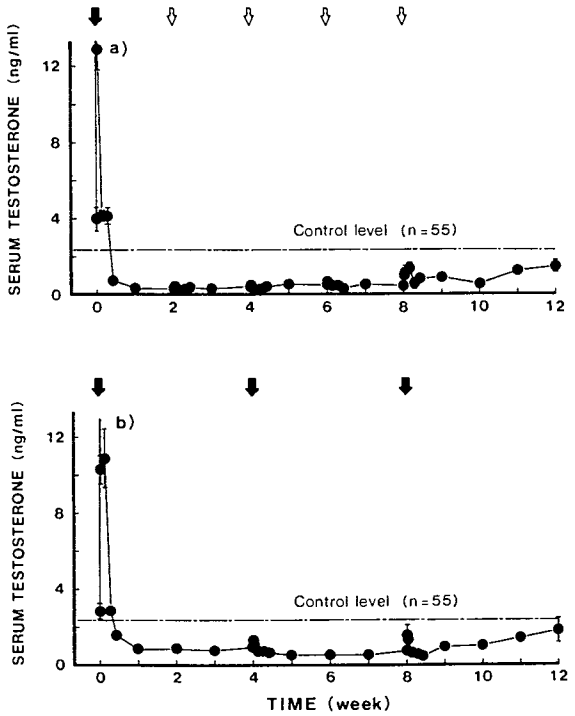

Fig. 6. Serum testosterone levels in rats after repeated s.c. injection of leuprolide microspheres at 2- or 4-week intervals. The doses were 1.35 (⬇) and 0.675 (⇩) mg/rat (10 weeks of age) of leuprolide acetate. Each point represents the mean ± SE of five rats. (a) $C_{3\ hr}$ = 27.1 ± 5.2 ng/ml; (b) $C_{3\ hr}$ = 15.0 ± 0.8 ng/ml.

formulation not only avoided this daily small response but also diminished the response just after repeated injection because of sustained drug release. Slightly stronger suppression was obtained with the treatment at 2-week intervals than at 4-week intervals. However, this suppression following the repeated treatment at 4-week intervals is sufficient. Thus, using this once-a-month depot formulation may not only eliminate the inconvenience of daily injections but also assure patient compliance and produce more reliable therapeutic effects.

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