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Immunization of Rams against Human Recombinant Inhibin α-Subunit Delays, Augments, and Extends Season-Related Increase in Blood Gonadotropin Levels[1]

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ABSTRACT

Adult Suffolk rams were immunized four times against the human recombinant inhibin α-subunit over a period of 80 days. Blood samples were collected at weekly intervals and serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were determined by radioimmunoassay procedures. The results show that season-related elevations of gonadotropin levels in immunized rams was delayed by 1 – 2 wk and, in these animals, it was more pronounced and extended than in vehicle-treated controls. Peaks of circulating testosterone were higher in control rams than in immunized animals. The capacity of the antisera to bind ^{125}I-labeled inhibin α-subunit increased significantly in each immunized animal within 30 days of treatment, even though neutralizing antibodies were detected with a rat pituitary cell culture bioassay in only one of the four immunized rams. Epididymal sperm reserves tended to be greater in immunized than in control animals. These results show that inhibin controls the release of FSH during the breeding season, thereby regulating spermatogenic activity; it may also exert its effect on testicular function by a local effect on Leydig cells, as evidenced by changes in serum testosterone profiles and increased serum LH levels in rams immunized against the inhibin α-subunit.

INTRODUCTION

Inhibin, by way of negative feedback, controls the synthesis and release of follicle-stimulating hormone (FSH) by the anterior pituitary (McCullagh, 1932; de Kretser et al., 1987). To study the physiology of inhibin, several investigators have chosen the approach of immunoneutralization of this gonadal glycoprotein hormone. In ewes, immunization against inhibin-enriched preparations of bovine follicular fluid leads to a marked increase in ovulation rate and elevated serum FSH levels (Henderson et al., 1984; O'Shea et al., 1984). Inhibin has been purified from reproductive tract fluids of several species (Vaughan et al., 1989), and DNA sequence analysis of the subunit genes has revealed considerable structural similarities between species. Nevertheless, species differences, although minor, in the amino acid sequence of the α-subunit prompted its use as an immunogen for neutralization of endogenous inhibin. When ewes were treated with the bovine or human recombinant inhibin α-subunit, there was a remarkable immunological response as evidenced by increased ovulation rate and elevated serum FSH levels (Forage et al., 1987; Findlay et al., 1989; Mizumachi et al., 1989).

Information on the feedback control of inhibin in males is derived largely from in vitro studies involving

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isolated Sertoli, pituitary, and Leydig cells; there are few studies on the interaction of inhibin with these cells based on in vivo results (de Kretser et al., 1987). In the present study, we immunized adult rams using the human recombinant inhibin α-subunit in the hope of increasing our understanding of the control and function of inhibin in males.

MATERIALS AND METHODS

Immunization of Rams

Nine adult Suffolk rams, 4 treated and 5 control animals, weighing between 40 and 65 kg, were maintained on pasture and hay ad libitum supplemented by 200 g/day of a mixture of oats and molasses. The immunogen used was the human inhibin α-subunit produced by recombinant DNA methods. The complementary DNA (cDNA) coding for the human testicular inhibin α-subunit precursor was isolated and characterized as described by Keinan et al. (1989). The portion of cDNA that contained the nucleotide sequence for the mature α-subunit peptide was subcloned and expressed in *Escherichia coli*. The N-terminal of the recombinant α-subunit (Ser-Thr-Pro-Leu-Met) did not differ from that of the native human inhibin α-subunit. Characteristics and amino acid sequence of the α-subunit have been reviewed elsewhere (see Mayo et al., 1986; Vale, 1988; Bardin et al., 1989). Finally, the recombinant α-subunit peptide was purified and its identity was verified by amino acid sequencing. Each dose of 100 μg of human recombinant inhibin α-subunit in vehicle (0.5 ml of 50 mM tris(hydroxymethyl)aminomethane [Tris]-4 M urea) was emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO) and injected i.m. into the hind legs. The immunogen was applied four times at intervals of 20, 30, and 30 days, starting 12 July (Day 55 of experiment) or well in advance of the breeding season. Control animals were treated with the vehicle only.

Collection and Assay of Serum Samples

Beginning on 19 May (Day 1 of experiment), five blood samples were collected from the jugular vein of each animal at hourly intervals once a week. The five serum samples from each of the 4 treated and 5 control animals were pooled separately and stored frozen until assayed. Levels of serum FSH, luteinizing hormone (LH), and testosterone were determined by radioimmunoassay (Sanford, 1985). The interassay coefficient of variation for FSH, LH, and testosterone was 7.9%, 9.9%, and 0.19%, respectively, and intraassay variability was 3.5%, 13.4%, and 8.4%, respectively.

The immunoresponse to the human recombinant inhibin α-subunit was determined by a ^{125}I-α-subunit binding assay using an approach similar to that described by Forage et al. (1987). Human recombinant inhibin α-subunit was labeled by the chloramin-T method (Ying et al., 1987). The reaction mixture was equilibrated with 30% (w/v) acetic acid on Sephadex G-25, and the radioactivity of the labeled tracer was adjusted to ~3×10^6 cpm/ml. Shortly before use, the tracer was diluted again with 6 volumes of 1 M Tris buffer (pH 9.0), and the concentration was adjusted to $50 - 100 \times 10^4$ cpm/ml with 0.05 M phosphate-buffered saline (PBS)-azide (pH 7.7)-0.1% bacitracin (ICN, Irvine, CA)-5 mM methionine-0.1% (3-[(3-cholamidopropyl)-dimethylammonio] 1-propane-sulfonate) (CHAPS). The tracer ($5 - 10 \times 10^4$ cpm) was added to 300 μl of diluted sample of antiserum (final conc., 1:400). After incubation at 20°C for 16 h, the antibody was precipitated by the addition of 100 μl of 0.1% (w/v) bovine gamma globulin-0.05 M PBS-azide and 500 μl of 25% (w/v) polyethylene glycol-0.05 M PBS-azide. The reaction mixture was then incubated at 20°C for 30 min. The tubes were centrifuged at $2500 \times g$ for 40 min and the pelleted precipitate was counted in a gamma counter. The results are expressed as the percentage cpm bound of added cpm minus assay background cpm.

The presence of neutralizing antibodies in serum was assessed by the bioassay procedure similar to that described by Scott et al. (1980), McLachlan et al. (1986), and Mizumachi et al. (1986). Serum samples, in 25-, 50-, and 100-μl volumes were adjusted to 100 μl with normal sheep serum and mixed with 200 μl charcoal-treated rete testis fluid (RTF) before incubation at 37°C for 1 h. The effects of large volumes of serum were eliminated by adding 100 μl normal sheep serum to RTF samples selected for standards. The reaction mixtures were then added separately to established rat pituitary cell cultures in 96-well plates and incubated at 37°C for 24 h. Accumulation of FSH in the medium was determined by radioimmunoassay using the NIDDK-rat FSH kit. The intra- and interassay coefficients of variation were 5.7% and 8.6%, respectively.

Determination of Sperm Reserves

At the end of the experiment (Days 162/163), the left testis was surgically removed from each of the treated

(4) and control (5) animals. To assess epididymal sperm reserves distal to the proximal corpus, the epididymis was carefully dissected out of its connective tissue and homogenized in 0.9% saline in a Virtis "23" blender (Gardiner, New York, NY). After dilution of the homogenate to the appropriate concentration, the sperm were counted in a hemocytometer. Sperm reserves were expressed per gram of testis, because testis weights between animals varied greatly (reviewed by Amann, 1981).

Statistical Analysis

The effects of season and treatment have been statistically evaluated by identifying the first samples (days) showing higher gonadotropin levels above two standard deviations of the mean during the preinjection period. Mean values and duration of elevation of the control animals were then compared with those of the immunized animals by analysis of variance. For testosterone, the number of episodic peaks above two standard deviations of the mean were identified and assessed for the entire experimental period.

The results of the bioassay have been statistically evaluated by the parallel line (2 × 2 point) method, which compares dose-response curves obtained in the presence of antiserum (apparent relative potency) with those resulting from incubation without antiserum (potency = 1) (Mizumachi et al., 1986).

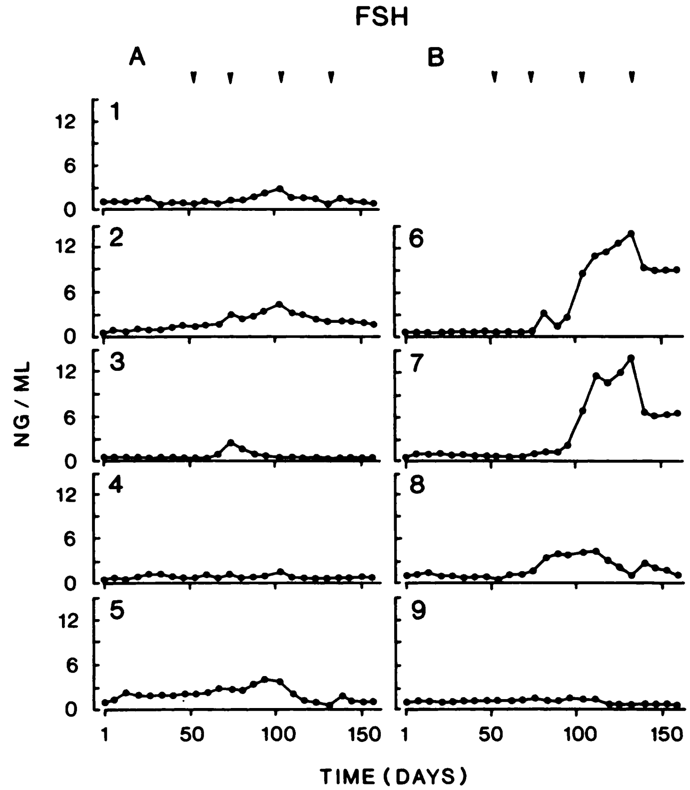

FIG. 1. Effect of immunization of rams against the human recombinant inhibin α-subunit on peripheral blood FSH levels. (*A*) Controls; (*B*) immunized animals. *Arrows*, times of injection of immunogen.

RESULTS

Serum Gonadotropin Levels

Figures 1A and 2A show that, in the control animals, the first significantly elevated blood FSH levels were observed between Days 63 and 91, and higher LH levels were detected between Days 70 and 77 of the experiment. In the immunized animals, the first elevated levels of FSH were recorded between Days 63 and 84 (Fig. 1B) and for LH on Day 84 or approximately 2 wk later than in the control rams. These season-related increases in circulating FSH and LH in control animals were 1- to 5-fold ($p<0.01$) and 1- to 7-fold ($p<0.01$), respectively, and elevated levels of FSH and LH persisted for 36 – 98 days or more and 50 – 91 days or more, respectively. Rams 4 and 5 were exceptional in that the former showed no significant increase in FSH concentration, whereas the latter exhibited no persistent increase in LH level. The seasonal effect on FSH and LH levels was much more pronounced ($p<0.01$) in immunized rams than in control rams (Figs. 1B, 2B). Thus, the concentration of FSH in the immunized rams increased up to 26 times and that of LH up to 25 times, so that by Day 106 – 127, levels of both gonadotropins were much higher ($p<0.01$) in immunized than in control animals. Ram 9 was exceptional amongst immunized animals in that FSH stimulation did not exceed that of control animals, even though there was an 8-fold increase in circulating LH. In immunized animals, in contrast to control animals, elevated FSH and LH levels persisted throughout the experiment.

Testosterone

There were no persistent differences in testosterone levels between control and immunized rams before the breeding season. However, after Days 50 – 55, coinciding approximately with the beginning of the breeding season, the blood testosterone profile of each control animal displayed a significantly higher peak (16 ± 2.6

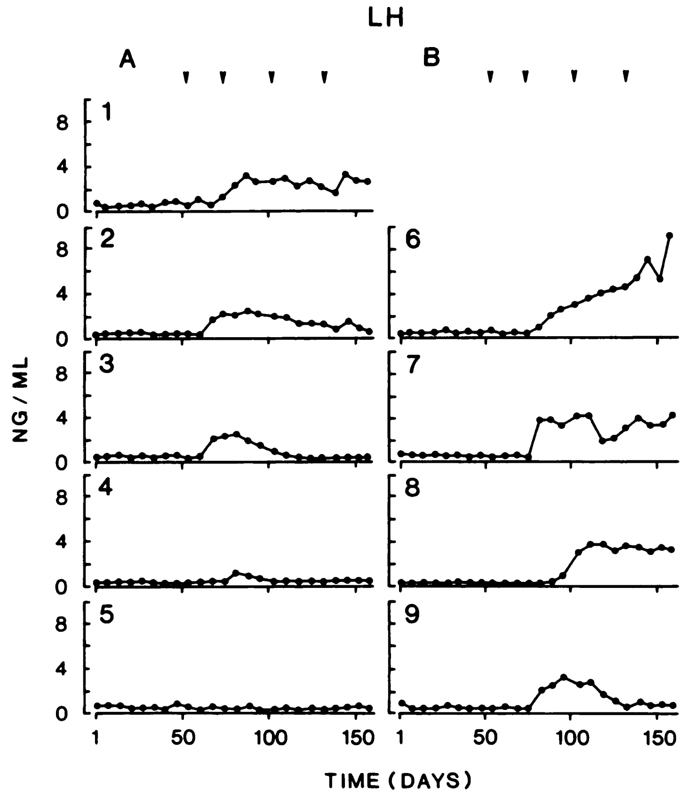

FIG. 2. Effect of immunization of rams against the human recombinant inhibin α-subunit on peripheral blood LH levels. (*A*) controls; (*B*) immunized animals.

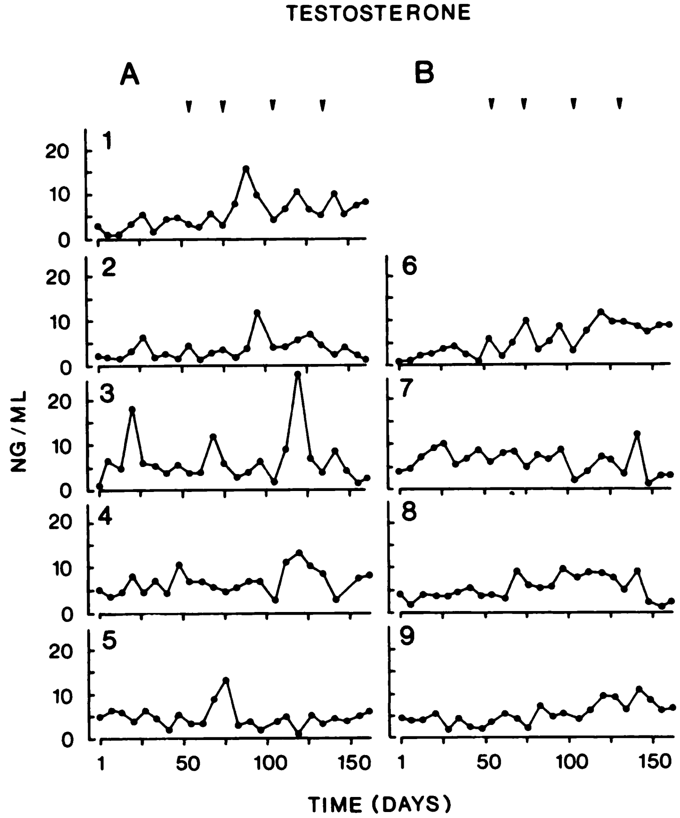

FIG. 3. Effect of immunization of rams against the human recombinant inhibin α-subunit on peripheral blood testosterone levels. (*A*) Controls; (*B*) immunized.

ng/ml vs. 11 ± 0.5 ng/ml, $p<0.01$) than was the case in the immunized animals whose profile was less variable (Fig. 3A,B).

Binding of ^{125}I-α-Subunit and Neutralizing Antibodies

The time course of ^{125}I-α-subunit binding to serum is shown in Figure 4. By Day 82, i.e. 7 days after the first booster injection, all immunized rams had developed high serum binding capacity. Mean maximum or near-maximum ^{125}I-α-subunit binding was observed on Day 113 or 8 days after the second booster injection, whereas binding of the tracer to serum of control animals was always marginal (range, 0.1% – 5.5%).

Immunoneutralizing antibodies were detected in one ram (Ram 6) after the third booster injection. Relative potencies for pre- and post (Day 142)-treatment were 0.74 and 0.19, respectively. This also was the most responsive animal as judged by elevation of gonadotropin levels (see Figs. 1B, 2B). However, the bioassay did not allow detection of lower titers of neutralizing antibodies presumed to be present in the remaining immunized animals.

Epididymal Sperm Reserves

The mean epididymal sperm reserve of the control rams was 1.19 ± (SE) 0.21 × 10^8 sperm/g of testis (range, 0.51 – 1.77 × 10^8 sperm/g testis). The sperm reserve of the immunized animals averaged 1.5 ± 0.34 × 10^8 sperm/g testis (range, 1.03 – 2.49 × 10^8 sperm/g testis). This represents a 26% difference, but because of the small number of experimental animals and variations between animals, it was statistically not significant ($p<0.1$).

DISCUSSION

Season-related increases in gonadotropin levels as described here have been well documented (Findlay et al., 1985), but the magnitude of the response to immunization against the human recombinant α-subunit was

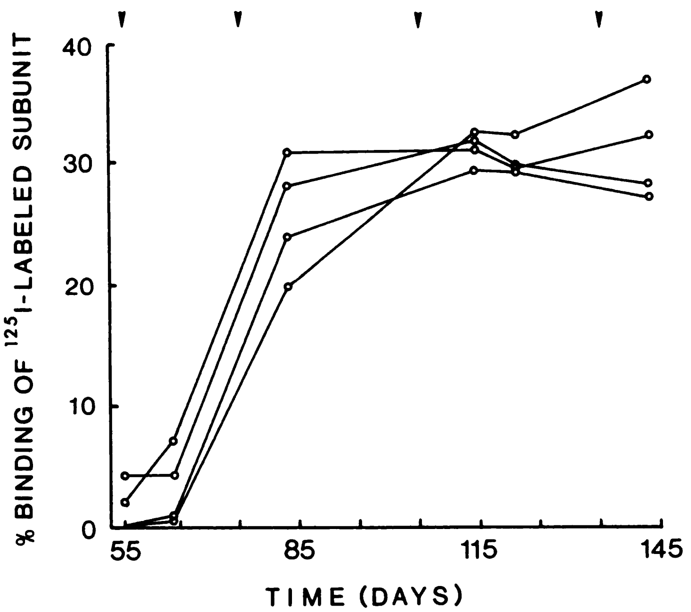

FIG. 4. Binding of ^{125}I-labeled inhibin α-subunit to diluted (1:400) serum of rams immunized against the human recombinant inhibin α-subunit at times shown.

unexpected. While such season-related increases in gonadotropin levels are apparently initiated by direct hypothalamic control of pituitary gonadotropin secretion, as had been demonstrated in castrated animals (Findlay et al., 1985), these effects are evidently amplified by immunosuppression of inhibin normally secreted by the testis. This demonstrates a direct feedback of inhibin to the hypothalamo-pituitary axis and the involvement of the nonsteroidal factor inhibin in controlling, at least partially, gonadotropin release. The fact that the magnitude of the response to immunization was greater in FSH than in LH would seem to support the theory that the response to inhibin of FSH-producing pituitary cells differs from that of LH-secreting cells. Studies with rat pituitary cells in vitro, for example, have shown that inhibin suppresses gonadotropin-releasing hormone (GnRH)-stimulated FSH and LH secretion equally well, but its effect on basal LH secretion is minor in comparison with that on basal FSH release (Farnworth et al., 1988). It is, therefore, not surprising that the response to the α-subunit treatment was greater for FSH than LH levels. Immunization of rams against human recombinant inhibin α-subunit clearly showed ^{125}I-α-subunit binding response in all treated animals; we have also demonstrated the presence of neutralizing antibodies by bioassay. It should be noted that neutralizing antibodies were found in only one treated animal, and it is interesting that this is the same animal with the highest elevations of gonadotropin levels. Forage et al. (1987) also found neutralizing antibodies in only 60% of bovine α-subunit-immunized ewes, all of which displayed higher ovulation rates than the controls; we have demonstrated a similar physiological response in ewes by applying an immunization regimen identical to that used in the present study (Mizumachi et al., 1989).

The delay observed in the onset of the seasonal effect in the immunized animals is difficult to explain, but it may be related to increased sensitivity to GnRH of the pituitary cells at the beginning of the breeding season (Findlay et al., 1989), and inhibin neutralization may delay activation of this mechanism. Similarly, delayed desensitization to GnRH of the pituitary cells could explain the prolonged seasonal effect in the immunized rams.

The fact that immunization against the inhibin α-subunit eliminated most the high peaks in the testosterone profile raises the possibility of a local effect of inhibin on Leydig cell function. It has been shown that inhibin is one of the Sertoli cell products that communicates with adjacent Leydig cells to modulate production of testosterone (Hsueh et al., 1987). Suppression of such testosterone peaks by inhibin α-subunit treatment may be one of the explanations for the high serum LH levels found in immunized rams.

The difference (26%) in epididymal sperm reserves was statistically not significant. Nonetheless, it is interesting that application of an identical immunization regimen to ewes resulted in a 4- to 5-fold increase in ovulation rate and elevated pre- and post-ovulatory serum FSH levels (Mizumachi et al., 1989). Thus, the physiological consequences of inhibin neutralization in rams seem to parallel the effect seen in ewes, namely, an increase in the production of germ cells.

In summary, immunization of rams against the human recombinant α-subunit delays, amplifies, and extends the season-related increase in serum gonadotropin levels, but the results are also indicative of a local inhibin effect on Leydig cell function.

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