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Relative ability of ovine follicle stimulating hormone and its β -subunit to generate antibodies having bionutralization potential in nonhuman primates

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Abstract. The relative ability of ovine follicle stimulating hormone and its β -subunit, two potential candidates for male contraceptive vaccine, to generate antibodies in monkeys capable of bionutralizing follicle stimulating hormone was assessed using *in vitro* model systems. Antiserum against native ovine follicle stimulating hormone was found to be highly specific to the intact form with no cross-reactivity with either of the two subunits while the antiserum against β -subunit of follicle stimulating hormone could bind to the β -subunit in its free form as well as when it is combined with α -subunit to form the intact hormone. Both antisera could block the binding of the hormone to the receptor if the hormone was preincubated with the antibody. However, the follicle stimulating hormone β -antisera could only inhibit the binding of the hormone partially (33% inhibition) if the antibody and receptor were mixed prior to the addition of the hormone, while antisera to the native follicle stimulating hormone could block the binding completely (100% inhibition) in the same experiment. Similarly antisera to the native follicle stimulating hormone was significantly effective in blocking (100%) response to follicle stimulating hormone but not the β -subunit antisera (0%) as checked using an *in vitro* granulosa cell system. Thus the probability of obtaining antibodies of greater bionutralization potential is much higher if intact hormone is used as an antigen rather than its β -subunit as a vaccine.

Keywords. Gonadotropins; hormone action; receptor.

Introduction

Immunoneutralization of endogenous follicle stimulating hormone (FSH) (using either passive or active immunization approach) has been used in this laboratory in the past as a means to gain insight into the mechanism of regulation of ovarian and testicular function by FSH both in the rat and in the primate (Moudgal, 1981; Sheela Rani and Moudgal, 1983; Moudgal and Dighe, 1985). Based on these studies we proposed developing ovine FSH (oFSH) as a contraceptive vaccine for use by the human male (Moudgal *et al.*, 1986). Chronic neutralisation of circulating FSH in adult male monkeys by specific antibodies has been shown to result in testicular dysfunction leading to infertility (Murty *et al.*, 1979). However, it was not clear from

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Abbreviations used: FSH, Follicle stimulating hormone; oFSH, ovine FSH; a/s, antisera; PRP, particulate receptor preparation; PMSG, pregnant mare serum gonadotropin; RIA, radioimmunoassay; hCG, human chorionic gonadotropin; LH, luteinizing hormone.

these studies how these antibodies act to bring about neutralization of FSH bioactivity *in vivo*—whether by sequestering and reducing peripheral FSH concentration and/or also by acting as an effective competitor of the receptor for binding FSH. Development of effective FSH vaccine is perhaps dependent upon the antigen under test producing antibodies capable of blocking FSH action at both the loci described above. The two candidate vaccines considered were intact FSH and its β -subunit. Since an antibody capable of blocking hormone-receptor interaction *in vitro* can be assumed to act as a good sequestering agent *in vivo* also, it was considered desirable to develop *in vitro* model systems wherein the ability of antibodies raised to different antigen to block hormone action can be studied. In the present study we have compared receptor binding inhibitory activity of an antiserum raised against native oFSH to that of an antiserum raised against oFSH β -subunit.

Materials and methods

Antisera

The antisera (a/s) used in these studies were raised in monkeys against highly purified oFSH (Sheela Rani and Moudgal, 1977) and its β -subunit (Sairam, 1979; Raj *et al.*, 1980). Information on the source and characterization of these a/s is provided.

Receptor preparation

The first part of the study was carried out using a particulate receptor preparation (PRP) prepared from pig granulosa cells (Sebok *et al.*, 1987). A pool of cells obtained during aspiration of pig follicular fluid was collected by centrifugation at 15,000 g for 30 min at 4°C, washed with 25 mM Tris HCl, pH 7.4, and homogenized in the same buffer using an all glass homogenizer. The suspension was finally lyophilized and stored at 4°C until used. Lyophilized preparations of the receptor have been kept stored for more than 5 years without loss of either [¹²⁵I]FSH or [¹²⁵I]hCG binding activity. Whenever necessary, suspension of this powder (20 mg/ml representing approximately 2 mg protein equivalent/ml) was prepared in 25 mM Tris-HCl, pH 7.4 containing 10 mM MgCl₂ and 0.1% bovine serum albumin (designated as RRA buffer).

Intact granulosa cells isolated from ovarian follicle of 23-day old female rats (Charles River, Canada) primed 48 h earlier with 15 IU/rat pregnant mare serum gonadotropin (PMSG) were also used as a source of the receptor. Following isolation, the cells were washed twice with Dulbecco's modified Eagle's medium and resuspended in a large volume of medium to provide approximately 250,000 cells per 0.1 ml aliquot. The cells were incubated with [¹²⁵I] oFSH in a total volume of 0.5 ml for different periods at 37°C in a Dubnoff metabolic incubator.

General methodology

Iodination of oFSH and subunits using carrier free [¹²⁵I]NaI was carried out

according to the procedure of Sairam (1978) and the specific activity of the preparation ranged from 50–80 $\mu\text{Ci}/\mu\text{g}$. Each incubation was performed in triplicate. The bound hormone was separated by centrifugation and the radioactivity in the pellet was determined using an autogamma spectrometer. Maximum binding ability of each [^{125}I] oFSH preparation was determined by incubating the tracer with large excess of PRP and was found to vary between 20–25% of the input. Whenever needed, the antigen-antibody complex was precipitated using the appropriate second antibody.

Progesterone in the granulosa cell incubation mixture was assayed using a radioimmunoassay (RIA) system standardized and routinely used in the laboratory (Kato *et al.*, 1983).

All a/s dilution indicated here are the initial dilutions, 100 μl of each dilution antiserum being used per tube and the total volume being 500 μl .

Results

Characteristics of the a/s used

Of the 3 a/s raised against intact oFSH initially screened for their affinity, specificity and capacity, one antiserum was chosen for comparison with antiserum against the β -subunit of FSH. Antisera I (raised in a monkey against intact oFSH-NRM 603) showed high affinity (as determined by Scatchard analysis of binding data) for oFSH (K_m – $1.1 \times 10^{10} \text{ M}^{-1}$) with a binding capacity of 130 μg oFSH/ml of serum. Antisera II (a pool obtained from monkeys immunized against FSH β -subunit-HGM 4) had relatively lower binding affinity for intact oFSH (K_m – $0.3 \times 10^9 \text{ M}^{-1}$), but the binding capacity (165 μg oFSH/ml) of this antiserum was almost similar to that shown by a/s I.

The cross reactivity of these two a/s with the subunits of oFSH was determined by incubating 0.1 ml of different dilutions of a/s with [^{125}I]labelled α - and β -subunits of oFSH. Both the a/s showed insignificant binding to the α -subunit and even this could be observed only at the lowest dilution (1:100 dilution). Binding of [^{125}I]FSH β -subunit to a/s II was high, 50% binding being observed at 1:7000 dilution. Relatively, 50% binding to intact [^{125}I] FSH was observed at a dilution of 1:2000. This is to be expected as number of binding sites in the free β -subunit can be masked once the β -subunit comes in association with the α to make native FSH. Antisera I was found to be more specific for native FSH and could bind labelled FSH β -subunit only at very low dilutions (50% binding of [^{125}I] oFSH β being observed at a dilution of 1:300). These results showed that a/s I was mostly specific for intact oFSH while a/s II could bind to intact oFSH through its β -subunit.

Effect of antibodies on oFSH-receptor interaction using a PRP

Preformed antibody [^{125}I] oFSH complex was unable to bind to FSH receptors (table 1). This was demonstrated by preincubating both the a/s (100 μl of 1:2000 dilution of each a/s) with [^{125}I]oFSH for 2 h at 37°C prior to addition of receptor preparation (2 mg PRP/tube) and continuing the incubation for additional 2 h at 37°C. This experiment essentially showed that either of the a/s at relatively the same

Table 1. Inability of [¹²⁵I]oFSH–Ab preformed complex to bind FSH receptors.

Incubated with a/s	cpm bound to receptor	
	mean ± SEM	Inhibition (%)
Nonspecific binding	2126 ± 85	—
Specific binding (No a/s)	7944 ± 53	—
Antisera I (to intact oFSH)	2001 ± 34	100
Antisera II (to oFSH β)	2753 ± 268	89

The complex (hormone-antibody) was formed by incubating [¹²⁵I]oFSH with 100 μl of 1:2000 diluted respective antiserum for 60 min at 37°C. At this time 2 mg of a lyophilized porcine granulosa cell receptor preparation in 100 μl of RIA buffer was added and incubation continued for 120 min. Amount of [¹²⁵I] oFSH bound to the particulate receptor was determined.

concentration, despite having different binding affinities were capable of forming a firm complex with FSH, preventing its subsequent binding to FSH receptors.

Ability of the two a/s to compete with the receptor for binding [¹²⁵I]FSH was determined by premixing either of the a/s (100 μl of 1:2000 dilution) with the receptor preparation (2 mg PRP/tube) prior to addition of [¹²⁵I]oFSH. Receptor bound radioactivity was determined at the end of 2 h incubation at 37°C. While a/s I was able to inhibit 100% the binding [¹²⁵I] oFSH to its receptor, a/s II was able to inhibit, at the same dilution, the binding by only 33% (table 2).

The effect of varying the concentration of the antibody or of the particulate receptor preparation on the ability of [¹²⁵I]oFSH to bind the receptor was next determined. Using a constant receptor concentration (2 mg in a total volume of 500 μl) binding inhibition of 50% could be obtained with 100 μl of 1:5000 diluted a/s I (figure 1). Conversely the ability of the receptor protein to effectively compete with the antibody for labelled oFSH increased as the receptor concentration was enhanced, 8 mg of receptor protein (in a total volume of 500 μl) being required to overcome the 50% inhibition of binding obtained when 100 μl of 1:2000 diluted a/s I was used. Though the antibody was an effective competitor of the receptor for binding labelled oFSH, it was unable to dissociate the [¹²⁵I]oFSH prebound to the PRP even when tested at a high concentration (100 μl of 1:1000). This was demonstrated by adding the antiserum after different association times (from 30–240 min) of the hormone with the PRP (data not shown).

Table 2. Relative ability of different a/s to prevent [¹²⁵I] oFSH from binding to the receptor.

Identification of the a/s	cpm bound to the receptor*	
	mean ± SD	Inhibition (%)
Nonspecific binding	3241 ± 50	—
Specific binding (No a/s)	13531 ± 513	—
Antisera I (to intact oFSH)	3229 ± 53	100
Antisera II (to oFSH β)	10084 ± 962	33

*Receptor preparation (2 mg/100 μl) and respective a/s (100 μl of 1:2000 dilution) were premixed before addition of [¹²⁵I]oFSH. Total volume of the reaction mixture was 500 μl and incubation time 2 h at 37°C. Results are mean of triplicate determinations. For other experimental details see foot note to table 1.

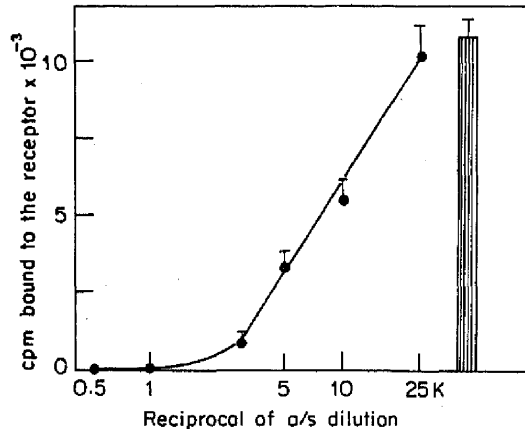


Figure 1. Effect of varying antibody concentration on the ability of ^{125}I oFSH to bind to receptor.

^{125}I oFSH (approximately 100,000 cpm) was added to tubes containing 2 mg of PRP and 100 μl of different dilutions of a/s I and incubated for 2 h at 37°C. At the end of incubation the receptor bound radioactivity was determined. The nonspecific binding determined by incubating ^{125}I oFSH with PRP in the presence of excess unlabelled oFSH was subtracted from the total binding observed. The bar indicates specific binding observed when ^{125}I oFSH was incubated with PRP only. The values are mean \pm SD of triplicate determinations.

Interaction of ^{125}I oFSH with its receptor in intact cells

Granulosa cells isolated from ovaries of PMSG primed rats were preincubated with ^{125}I oFSH for 30, 60 and 120 min at 37°C. After centrifugation and resuspension in a fresh medium, the cells were reincubated for 120 min in the presence or absence of added a/s. It is evident from figure 2 that a significant portion of the bound hormone (59% reduction in binding, $P < 0.001$) was released by a simple expedient of incubation of the cells in a hormone free medium. Addition of antiserum I but not a/s II to the incubation medium facilitated further discharge of the bound hormone (82% reduction in the bound hormone). The experiment also showed that association time of the ^{125}I oFSH with the granulosa cell had apparently no effect on the ability of the cell to discharge the bound hormone. It was also observed that antibody could dissociate receptor-bound hormone in the intact cells while it was unable to do so with particulate receptor (table 3).

Ability of FSH a/s to disrupt FSH stimulated steroidogenesis in granulosa cells

Incubation of rat granulosa cells with 50 ng FSH in the presence or absence of 10 μl of normal monkey serum resulted in stimulation of production of progesterone. Antiserum I was capable of inhibiting FSH response almost totally (98–100%) irrespective of whether it was preincubated with FSH or added subsequent to mixing cells and FSH in the incubation medium (figure 3). Antiserum II, in contrast, was only partially effective in blocking FSH response (48–55% reduction in progesterone produced, $P < 0.01$) and preincubation of FSH with the FSH β -antibody did not enhance the inhibition (figure 3).

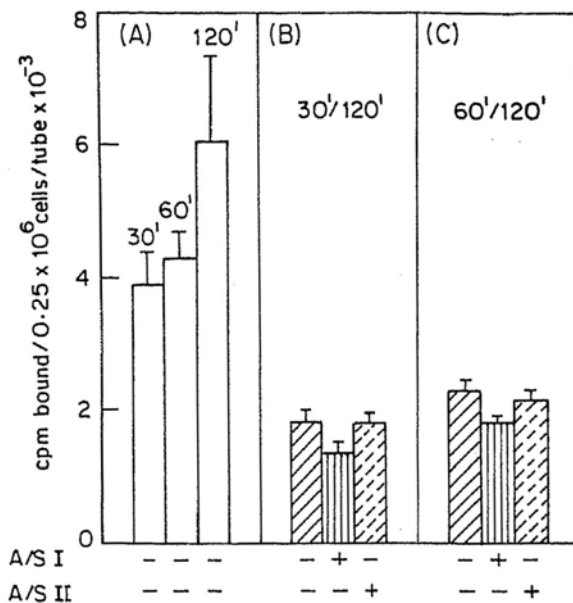


Figure 2. Ability of a/s to oFSH and its β -subunit to dissociate [125 I] oFSH bound for different periods to the rat granulosa cells.

Granulosa cells were incubated at 37°C for 30 and 60 min with [125 I]oFSH (1st incubation). The cells were centrifuged and binding was determined (panel A). The cells were resuspended in fresh medium (0.5 ml) containing 10 μ l of undiluted a/s I or II and reincubated for 120 min at 37°C (2nd incubation). At the end of incubation radioactivity bound to the cells was determined. Panels B and C show the results when the 1st incubation was for 30 and 60 min respectively.

Table 3. Ability of a/s to discharge [125 I] o F S H bound to the receptor.

Receptor preparation	cpm bound to receptor	
	No a/s	With a/s I*
Particulate	4990 \pm 455	5321 \pm 220
Granulosa cells	4338 \pm 417	1371 \pm 120**

The labelled hormone was preincubated with the receptor Preparation/cells for 1 h at 37°C prior to addition of a/s and continuing incubation for additional 2h. The receptor/cell bound radioactivity was determined. The results are mean \pm SD of triplicate determinations.

* Incubated with 10 μ l of undiluted a/s.

**Significantly different from controls ($P < 0.001$).

The necessity of having FSH in the medium throughout the period of incubation and ability of the a/s to stop the continued responsiveness of the cell to the bound FSH was next tested. The result (figure 4) show that when granulosa cells preincubated with FSH for 2 h were centrifuged and the medium replaced with fresh medium and incubation continued for additional 2 h, a significant reduction in progesterone production over the appropriate controls (52–55% , $P < 0.01$) was observed. Addition of a/s I to the medium during second incubation was effective in

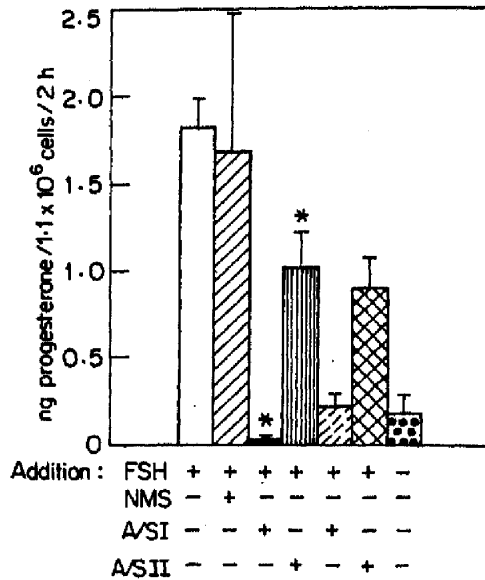


Figure 3. Ability of FSH a/s added at the beginning of the incubation on response of the granulosa cells to FSH.

Granulosa cells were incubated with either 10 μ l NMS or a/s I or II in the presence of oFSH (50 ng) for 2 h at 37°C and progesterone secreted into the medium was estimated by RIA.

*FSH in these cases was preincubated with a/s for 30 min prior to addition of cells.

further inhibiting progesterone production (from 52–78%). The a/s II, however, was once again ineffective in blocking steroidogenesis stimulated by the bound hormone (figure 4).

Discussion

The primary objective of the current study was to understand whether antibodies capable of sequestering FSH circulating *in vivo* are also able to inhibit FSH response *in vitro* and if so the mechanism by which this is achieved. All studies carried out hitherto on bionutralization of endogenous FSH have used either well characterized polyclonal antibodies or in active immunization studies, a characterized antigen. The chances of obtaining bionutralization *in vivo* is perhaps greater if more than one hormonal epitope is bound by its specific antibody. These determinants need not necessarily be those that interact with the receptor, as steric hindrances due to Ag-Ab complex at more than one locus could prevent the receptor recognition domain of the hormone from associating with the receptor.

The results of the present study have clearly shown that polyclonal bivalent antibodies do make excellent competitors of the receptor for binding to the hormone. Of the 4 a/s initially screened two were chosen for detailed study as they exhibited specific but different characteristics. It should be noted that both a/s I and II have been demonstrated in earlier studies to neutralize the bioactivity of FSH *in vivo* (Murty *et al.*, 1979; Moudgal *et al.*, 1986, 1988; Raj *et al.*, 1980). Antiserum I by virtue of its negligible binding to the free α - and β -subunits compared to its binding to intact oFSH could be classified as conformation specific. Antiserum II, in

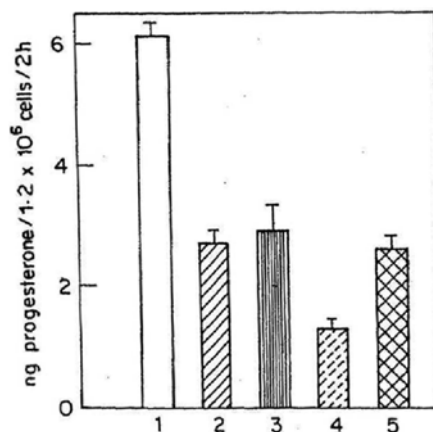


Figure 4. Ability of FSH a/s to disrupt ongoing steroidogenesis by granulosa cells under FSH stimulation.

The granulosa cells were preincubated with 50 ng FSH for 2 h. At the end of this incubation the cells were centrifuged, supernatant aspirated and the cells resuspended in medium (0.5 ml) containing either no addition, or with 10 μ l each of NMS or a/s I or II and incubated for a further period of 2 h at 37°C. Progesterone secreted into the medium was estimated by RIA. Progesterone produced when FSH was incubated with cells for 2 h served as controls. The values are mean \pm SD of triplicate determinations. (1), Controls (cells incubated with FSH for 2 h); (2), medium; (3), NMS; (4), a/s I; (5), a/s II.

contrast, by binding to the FSH β -subunit both in its free and combined state (intact FSH) showed that it is directed to specific sites in the β -subunit only. Though the binding capacity of a/s II was similar to a/s I, its binding affinity was relatively lower. While both the a/s used in this study were able to block completely the binding of labelled hormone to the particulate receptor preparation if the hormone was preincubated with the a/s, underscoring their *in vivo* sequestering property, their behaviour differed if the two binding entities (antibody and receptor) were mixed prior to the addition of [¹²⁵I]oFSH (table 2). It appears from this that the inhibition of binding was dependent more on their binding affinity than capacity. However, affinity may not be the only factor determining the inhibition potency as one of the other a/s raised to intact oFSH having nearly the same affinity ($\sim 0.5 \times 10^9$ M) as a/s II, proved in competitive experiments to be as good an inhibitor of [¹²⁵I]FSH binding to the receptor as a/s I (data not shown). Poor inhibitory capacity can also then be a reflection of epitope specificity. Though the β -antiserum (a/s II) can be expected to have antibodies to a number of epitopes specific to its free state, it is possible that a majority of these epitopes are either masked or altered once the β -subunit recombines with the α to form native FSH. The epitopes recognized by a/s II in the whole hormone are perhaps those which are also directly recognized by the receptor resulting in only partial inhibition of the response. In the case of a/s I, however, binding of the antibody to conformationally specific site(s) should be preventing the association of the hormone with the receptor. It is interesting that the relative inability of the FSH β -a/s (a/s II) to block binding or response to FSH observed here is very similar to that exhibited by human chorionic gonadotropin (hCG) β -a/s in our earlier studies on hCG-receptor interaction (Dighe and Moudgal, 1981, 1983). We had postulated, based on those

data, that initial recognition of hCG/luteinizing hormone (LH) by its receptor is occurring through sites specifically located in the β -subunit. The current study shows that this could be true for FSH also. It appears that for an antibody to be an effective inhibitor of hormone response it should bind site(s) involved in either direct interaction of the hormone with the receptor or to those located close to such sites. All the same in the present study the relative greater efficiency of the antiserum to intact FSH in competing with the PRP for binding of labelled hormone or blocking response of cells to FSH is perhaps due to antibodies binding the hormone at more than one loci/epitope making it difficult, because of steric consideration, for the hormone to associate with the receptor.

Another interesting observation that has emerged from the present study is that the interaction of FSH with its receptor varies depending upon the type of receptor used. While its binding to the particulate receptor preparation is tight even at early association times, its binding to cell-associated receptor is loose and a significant amount of the bound hormone dissociates upon incubating the cells in a hormone free medium. Unlike in the case of hCG where exposure of cells to the hormone for as short as 15 min is adequate to provide a full 2 h response (Dighe and Moudgal, 1983), continued association of FSH with the cellular receptor is essential for it to produce a sustained response. In this respect, oFSH appears to resemble PMSG and rat LH (Sheela Rani and Moudgal, 1985).

In conclusion, it can be stated that all polyclonal a/s capable of binding the hormone *per se* (radiolabelled or otherwise) need not necessarily be good competitors of hormone binding to the receptor. For complete bionutralization *in vivo*, however, the antibodies in addition to being able to sequester the circulating hormone with high affinity should perhaps be also effective competitors of hormone binding to the receptor. Using the above criteria we believe intact oFSH rather than its β -subunit could turn out to be a better contraceptive vaccine. Studies carried out so far have shown that active immunization with intact oFSH results in antibodies which block binding of FSH to its receptors. These antibodies in turn cause infertility in the primate without having any effect on LH dependent functions underscoring the specificity of the vaccine (Moudgal *et al.*, 1986, 1988).

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