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Circulating immunoglobulins that inhibit the binding of follicle-stimulating hormone to its receptor: a putative diagnostic role in resistant ovary syndrome?

Violeta A. Chiauzzi*, Leonardo Bussmann*, Juan Carlos Calvo*†, Victoria Sundblad* and Eduardo H. Charreau*†

**Instituto de Biología y Medicina Experimental (IBYME), Buenos Aires, Argentina and* †*Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

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OBJECTIVE To evaluate the presence of circulating immunoglobulins that inhibit FSH binding to its receptor (Ig-FSHR) in patients with premature ovarian failure (POF).

DESIGN Non-randomized study. Blood sampling for determination of circulating immunoglobulins.

PATIENTS Two hundred and forty-seven patients with POF and 60 normally menstruating women (controls).

MEASUREMENTS Circulating immunoglobulins that inhibit FSH binding to its receptor were assessed by FSH-binding inhibition assay.

RESULTS Twenty-three out of 247 women with POF presented circulating immunoglobulins that inhibit FSH binding to its receptor. These patients had been previously diagnosed as ROS. Sixty control subjects proved negative.

CONCLUSION Determination of the presence of circulating immunoglobulins that inhibit FSH binding to its receptor could be instrumental in diagnosing the gonadotropin resistance ovary syndrome.

Premature ovarian failure (POF) is defined as the complete cessation of menses before the age of 40 and is characterized by primary or secondary amenorrhoea, hypoestrogenism and elevated gonadotropin serum levels. The level of FSH (> 40 mUI/ml) is the hallmark for diagnosis (Yen *et al*., 1972). Also, ovaries present different follicular development: in some cases, there is total depletion of ovarian follicles, hence a permanent loss of ovarian function; in others, follicular structures are still preserved and therefore recovery of ovarian function, either spontaneous or induced, might be possible (Hoek *et al*., 1997). POF affects almost 1% of the Western female population (Coulam *et al*., 1986). This syndrome is very heterogeneous and has a multicausal pathogenesis, but aetiologies remain mostly unknown. There is accumulating evidence indicating that autoimmunity may be involved in idiopathic POF; moreover, POF occurs frequently in patients with two or more associated autoimmune diseases (Coulam & Ryan, 1979; LaBarbera *et al*., 1988; Hoek *et al*., 1997). Furthermore, several investigators have found circulating antibodies to human ovarian tissue in the sera from patients with POF (Kamp *et al*., 1974; Board *et al*., 1979; Coulam & Ryan, 1979; Elder *et al*., 1981).

Resistant ovary syndrome (ROS) is proposed as a follicular form of POF, with ovaries in which numerous morphologically normal primordial follicles are present (Jones & de Moraes-Ruehsen, 1969; Fox, 1992; Hoek *et al*., 1997). Most follicles show no evidence of development, though a few will have attained the preantral stage, while occasional follicles will have developed into, but not surpassed, the antral stage. In cases of secondary amenorrhoea, stigmata of previous ovulation are usually present (Fox, 1992). Because in ROS the follicular structures are still preserved, recovery of ovarian function, either spontaneous or induced, might be possible.

Antireceptor antibodies have been implicated in the pathogenesis of several autoimmune disorders, such as myasthenia gravis, Graves' disease and the insulin-resistant form of diabetes mellitus (Patrick & Lindstrom, 1973; Manley *et al*., 1974; Flier *et al*., 1975). In previous studies on two patients with ROS and myasthenia gravis (Chiauzzi *et al*., 1982; Escobar *et al*., 1982), we demonstrated the presence of circulating immunoglobulins that inhibited FSH binding to its receptor (Ig-FSHR), by blocking the FSH-receptor (FSHR) itself or a receptor-related membrane domain. The presence of these antibodies could explain the gonadotropin resistance in these patients.

In the present study, we evaluated the presence and the inhibitory mechanism of circulating Ig-FSHR in 247 patients with POF.

Materials and methods

Patients

A study was carried out in 247 patients with POF, aged 14–38 years old, all of them with normal 46,XX karyotype. From

Correspondence: Violeta A. Chiauzzi, MD, Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, C1428ADN, Buenos Aires, Argentina. Tel: (54) 11-4783-2869; Fax: (54) 11-4786-2564. E-mail: chiauzzi@dna.uba.ar

Table 1 Clinical findings of patients studied

Patients (*n*)	Diagnosis	*n*	Amenorrhoea, *n* (%)		Secondary amenorrhoea		Previous pregnancies *n* (%)	Other autoimmune disease
			Primary	Secondary	Age at last menstruation	Menstrual history *n* (%)		
247 POF	Not classifiable	224	40 (18%)	184 (82%)	20–36 years old	A. 110 (60%) B. 55 (30%) C. 15 (8%) D. 4 (2%)	52 (23%)	52 autoimmune thyroiditis 5 rheumatoid arthritis 2 vitiligo 2 SEL 1 Addison's disease 1 diabetes mellitus
	ROS	23	8 (35%)	15 (65%)	14–30 years old	A. 9 (60%) E. 6 (40%)	2 (9%)	10 myasthenia gravis 2 rheumatoid arthritis 3 autoimmune thyroiditis

A, eumenorrhoea before the onset of POF; B, episodes of eumenorrhoea/oligomenorrhoea before the onset of POF; C, oligomenorrhoea since menarche; D, irregular cycles with polymenorrhoea before the onset of POF; E, irregular cycles with episodes of oligomenorrhoea and/or amenorrhoea before the onset of POF.

1982 to 2001, serum was obtained from diagnosed patients who were derived from several hospitals to our laboratory for determination of circulating Ig-FSHR.

Patients had been characterized as POF due to amenorrhoea for over a year starting before the age of 40, with elevated serum FSH levels (> 40 mIU/ml) in two consecutive determinations. Serum LH concentrations were above 20 mIU/ml (normal follicular phase levels: 2–10 mIU/ml). Plasma 17β–oestradiol was under 15 pg/ml (normal follicular phase levels: 20–120 pg/ml). Further clinical characteristics are given in Table 1.

Sixty-eight patients, studied before 1992, had undergone ovarian biopsy (general consensus that ovarian biopsy was clinically indicated for POF patients was by then still accepted (Fox, 1992). A total of 71% of these patients ($n = 48$) had shown stroma with either atretic follicles or with no follicles at all. The remaining 20 biopsies had evidenced the presence of numerous cortical primordial follicles, which had led to the diagnosis of ROS. One hundred and seventy-nine patients had not been subjected to ovarian biopsy. In most of these patients, ultrasound had evidenced small ovaries (average: $2{\cdot}4 \pm 2{\cdot}1$ cm^3) when visualized. Low follicular activity had been identified in some of the visualized ovaries. Three patients had been diagnosed as ROS due to an ultrasound examination that had shown ovaries sized $14{\cdot}2 \pm 3{\cdot}6$ cm^3 (normal size range: 6 ± 3 cm^3) with hyperechogenic stroma and with numerous small follicular images (< 3 cm^3) at the periphery.

Diagnosis of myasthenia gravis was based on clinical findings and on determination of acetylcholine receptor (AChR) antibody as described by Vincent & Newsom-Davis (1985). Ten out of the 247 POF patients presented an AChR antibody titre ranging from 40 to 280 nm/l (normal range: < 0·3 nm/l). In addition, 55 POF patients presented autoimmune thyroid disease with highly positive sera for thyroglobulin and/or thyroid peroxidase autoantibodies.

All patients included in this study appeared to be phenotypically normal on physical examination for height, weight and habits. Association with other autoimmune disorders had also been evaluated (Table 1).

Sera from 60 normally menstruating women in the same age range (average 28 years old) with no evidence of autoimmune disease were used as controls. Neither patients nor control subjects were receiving steroids at the time of blood sampling.

The protocol was approved by the Instituto de Biología y Medicina Experimental Institutional Review Board. Informed consent was obtained from all patients and controls.

Determination of circulating immunoglobulins that inhibit FSH binding to its receptor

Immunoglobulin fractions were precipitated from all sera studied, using 0–50% sodium sulphate saturation. Precipitates were purified by protein A-Sepharose as described (Chiauzzi *et al*., 1982), and tested for their inhibitory activity on a FSH-binding assay.

Radioligand-receptor assay for FSH was performed using testis homogenates from immature rats as source of FSHRs, as previously described (Chiauzzi *et al*., 1982; Escobar *et al*., 1982). Briefly, 15-day-old rat testis homogenates diluted 1 : 4 wt/vol, in Dulbecco buffer containing 0·1% bovine serum albumin (DPBS-BSA), were centrifuged at 27 000 g and pellet was resuspended in DPBS-BSA to obtain 4 mg protein/ml of suspension. This fraction was used as source of FSHRs. One hundred microlitres (400 µg) of membrane preparations, 50 µl DPBS-BSA with 20 000–100 000 cpm [^{125}I]-hFSH and 100 µl of DPBS-BSA with or without an excess of unlabelled hormone, were incubated

for 16 h at 24 °C. Each sample was diluted with 2 ml of ice-cold DPBS-BSA and centrifuged at 6000 *g* for 30 min. The amount of labelled hormone bound to its receptor site was determined by removing the supernatants and counting the washed pellets in an automatic γ-spectrometer with a counting efficiency of about 70%.

To test the presence of circulating inhibitory activity of FSH binding, the purified immunoglobulin fractions (up to 400 µg protein) were incubated with 100 µl (400 µg) of membrane preparations for 10 h at 4 °C. After the addition of [^{125}I]-hFSH and DPBS-BSA with or without an excess of unlabelled hormone, the reaction was allowed to proceed as described above.

To evaluate the specificity of the binding inhibition further, the blocking effect of Ig-FSHR sera was analysed in a radioligand-receptor assay for PRL, using membrane preparations of rat ventral prostate (Charreau *et al*., 1977), and in a radioligand-receptor assay for LH/hCG, using interstitial cell membranes from mature rat testis (Charreau *et al*., 1978).

Binding studies

Affinity constant was calculated for the IgG fractions isolated from the sera of 10 patients, randomly selected out of 23. Varying amounts of iodinated hFSH (10^{-11}–10^{-9} M) were added to incubation tubes containing a constant amount of purified immunoglobulin (400 µg), plus or minus a 100-fold excess of cold FSH for assessment of nonspecific binding. Binding data were converted into Scatchard (1949) plots. In another set of experiments, varying amounts of purified IgGs obtained from patients' sera were added to a constant, close to saturation amount of iodinated hFSH, and the percentage of binding inhibition was calculated. A logit–log plot was constructed from binding data and the amount of IgG fraction needed to reduce FSH binding by 50% was thereafter calculated and considered as the ED_{50}, which was used for the monthly profile analyses. The affinity constant for the IgG fraction was calculated from the Scatchard plot data, using the method described by Best-Belpomme & Dessen (1973).

In vitro *bioassays*

Ovaries were obtained from 24- to 25-day-old female Sprague–Dawley rats, and granulosa cells were prepared as described by Barañao *et al*. (1991). Cells (10^6) were preincubated for 1 h in Dulbecco's modified Eagle's medium (DMEM; 4·5 g glucose/l)/Ham's F12 (1 : 1, v/v)/HEPES (10 mM), with 400 µg of purified immunoglobulin fractions from ROS patients and from 12 control subjects, in a final incubation volume of 2 ml. At the end of the preincubation period, 2–1000 ng of ovine FSH (20 IU/mg, provided by the National Pituitary Agency, NIH, Bethesda, MD, USA) were added to the corresponding tubes, and incubation was allowed to proceed for an additional 3-h period. Finally, E_2 was measured by radioimmunoassay (RIA) as described (Chiauzzi *et al*., 1982), in a small aliquot of the incubation medium.

Leydig cells were obtained from adult male Sprague–Dawley rat testis, as described by Mendelson *et al*. (1975). Collagenase-dispersed cells (10^6) were preincubated in 2 ml of medium 199 (Difco, Detroit, MI, USA) containing 0·1 M methyl-isobutyl xanthine (MIX) with 400 µg of serum globulin fractions from ROS patients and from 12 control subjects, for 2 h at 34 °C. After the addition of 0·015–20 ng of hCG (11 000 IU/mg, provided by the National Pituitary Agency, NIH), incubations were allowed to proceed for an additional 2-h period. After incubation, tubes were centrifuged and supernatant collected for testosterone determination using a specific RIA, without extraction (Tesone *et al*., 1976).

Results

Circulating Immunoglobulins that inhibit FSH binding to its receptor

We had already characterized a circulating FSHR binding inhibitor in two patients with hypergonadotropic amenorrhoea and myasthenia gravis. The inhibitor was found to behave as an immunoglobulin according to several criteria (Chiauzzi *et al*., 1982; Escobar *et al*., 1982).

In the present study, we found that 23 out of 247 patients with POF presented circulating Ig-FSHR. These patients had been previously diagnosed as ROS, 20 of them by ovarian biopsy and the remaining three by ultrasonography. Figure 1 shows the ED_{50} for the Ig-FSHR corresponding to 20 of these patients, evidencing that the titres vary considerably among them. Sera from the 60 control women did not evidence these circulating antibodies (Fig. 2).

A strong association of POF syndrome to autoimmune disorders was observed, mainly to autoimmune thyroiditis (22·7%),

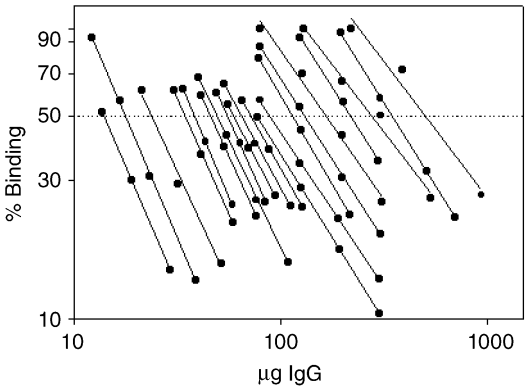

Fig. 1 Percentage of FSH bound to its receptor in the presence of varying amounts of patients' IgG fractions. ED_{50} corresponding to IgG fraction from each Ig-FSHR patient (dotted line) was calculated as the amount of IgG necessary to reduce total FSH binding by 50%.

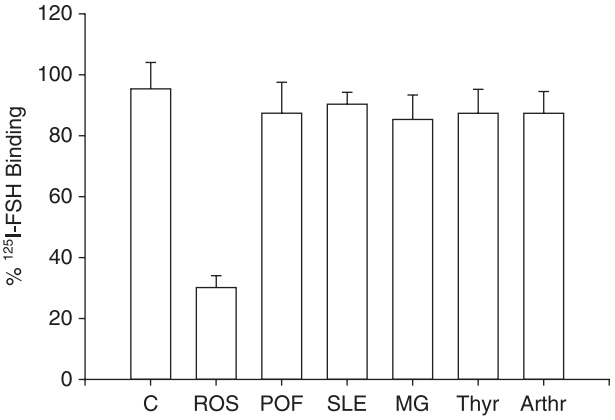

Fig. 2 FSH-specific binding to preincubated membranes with different IgG fractions (400 μg protein). C are serum IgG fractions from 60 control subjects, ROS are serum IgG fractions from the 23 ROS patients, POF are serum IgG fractions from 224 nonclassifiable POF patients. Purified immunoglobulin fractions from normally menstruating women with other autoimmune disorders are shown: SLE, two women with systemic lupus erythematosus; MG, 28 women with myasthenia gravis; Thyr, five women with autoimmune thyroid; Arthr, five women with rheumatoid arthritis. Results are expressed as the percentage of total FSH specific binding to membranes preincubated in the presence of buffer. Data are expressed as mean ± SEM.

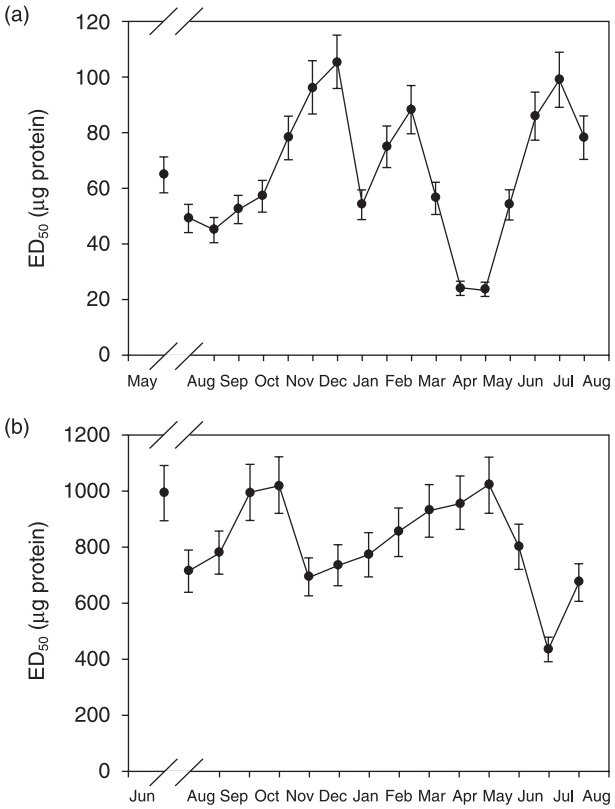

Fig. 3 Monthly profile for IgG secretion corresponding to ROS patients O (a) and H (b) from blood drawn from the patients once or twice a month. Data are expressed as mean of ED_{50} ± SEM.

rheumatoid arthritis (RA, 2·8%), systemic lupus erythematosus (SLE, 0·8%) and vitiligo (0·8%). However, sera from normally menstruating patients with autoimmune disorders, such as SLE, RA and autoimmune thyroid disease, did not evidence circulating Ig-FSHR (Fig. 2). In addition, 10 out of the 23 patients who presented circulating Ig-FSHR and who had been diagnosed as ROS by ovarian biopsy, also evidenced myasthenia gravis. Nevertheless, 28 serum IgG fractions from normally menstruating women with myasthenia gravis did not evidence these circulating antibodies (Fig. 2).

Although in a previous publication we demonstrated that the circulating Ig-FSHR corresponds to IgG molecules, to ascertain that the blocking effects of the patients' samples were indeed due to IgG, and not to a contamination with other blocking factors, experiments were carried out to neutralize the blocking effects of the preparations with rabbit antihuman IgG. Increasing amounts of anti-IgG were incubated with ED_{50} concentrations of the circulating inhibitor from the 23 Ig-FSHR patients, and after centrifugation, supernatants were assayed for FSH binding inhibition. Antihuman IgG neutralized the FSH binding inhibitor activities of all patient preparations (data not shown).

Variation of immunoglobulin titre along the course of the disease

There is evidence that in several autoimmune diseases, the autoantibody titre changes with time (Salvi *et al*., 1988). Serial serum samples from two out of the 23 Ig-FSHR patients, both diagnosed as ROS by ovarian biopsy, were tested monthly for a period of 2 years for Ig-FSHR. Figure 3 illustrates the monthly profiles corresponding to patients O and H; both had developed secondary amenorrhoea and then showed myasthenia gravis associated to ROS.

Although throughout the study the serum samples maintained anti-FSHR activity, their titres waxed and waned with time, showing a fluctuating course of the disease.

Mechanism of the inhibition of FSH binding

Figure 4 shows the Scatchard plots obtained from the analysis of the inhibition data from the FSH ligand binding experiments, using purified IgG fractions from serum of 10 out of the 23 Ig-FSHR patients. These results clearly demonstrate that the inhibitory activity of immunoglobulin fractions can be classified into two groups. One group exhibited a marked reduction in total free binding sites when receptors were exposed to immunoglobulin fractions. The apparent K_i was $2{\cdot}94 \times 10^{12}$ M^{-1}, a thousand times

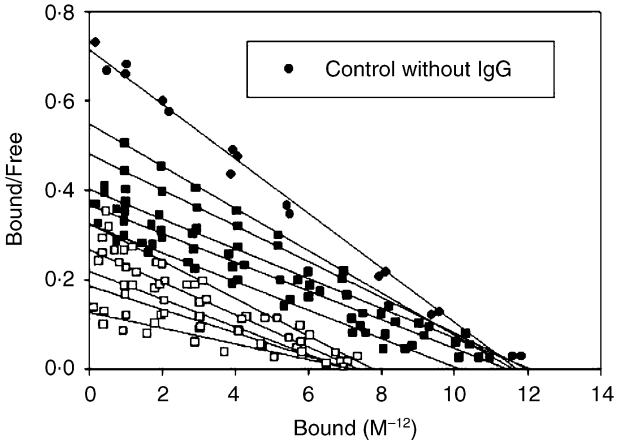

Fig. 4 Scatchard plots derived from saturation curves for FSH binding to testicular membranes. IgG fractions were precipitated with 0–50% sodium sulphate and purified by protein A-Sepharose. 400 µg IgG obtained from sera of 10 Ig-FSHR patients were added to each incubation tube. Iodinated FSH was added between 10^{-11} and 10^{-9} M. Two sets of IgG fractions were observed, one with binding characteristics similar to FSH affinity constant for its receptor, and another group with an affinity constant 1000 times higher, rendering this latter one irreversible. Control incubation was performed in the absence of immunoglobulin fraction. ●, control binding; ■, patients with low-affinity antibodies; □, patients with high-affinity antibodies.

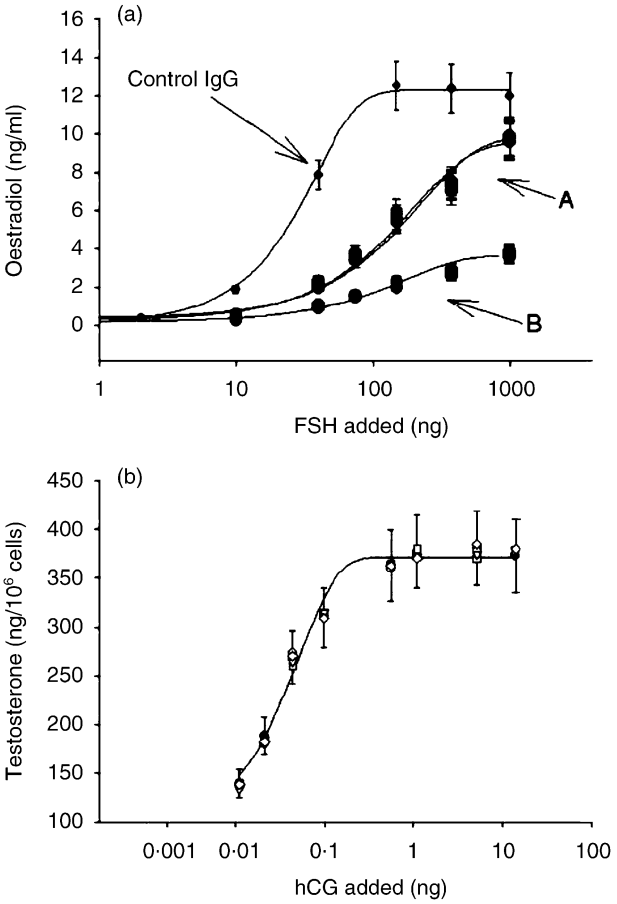

Fig. 5 *In vitro* bioassays. (a) Oestradiol production from granulosa cell preparations after stimulation with varying amounts of ovine FSH in the presence of 400 µg of IgG fractions. Sample illustration of all the population studied, with results corresponding to 10 Ig-FSHR patients and five controls. Lines A show the results corresponding to IgG fractions (five patients) with affinity constant for FSHR similar to the native hormone. Lines B show the results of IgG fractions (five patients) with 1000 times higher-affinity constant for FSHR as compared to the native hormone. Data are expressed as mean ± SEM. (b) Testosterone production from isolated Leydig cells, after 4 h incubation with increasing amounts of hCG, in the presence of 400 µg of IgG fractions. Sample illustration of all the population studied, with results corresponding to five Ig-FSHR patients and five controls. No differences were observed between curves (control versus Ig-FSHR patients). Data are expressed as mean ± SEM.

higher than the affinity constant for FSHR-binding interaction ($K_a = 3{\cdot}86 \times 10^9\ M^{-1}$). The foregoing suggests an 'irreversible' nature of the inhibitory effect. The inhibitor may therefore bind tightly to the receptor itself or to a receptor-related membrane domain. The other group of patients presented a 'reversible' mechanism of inhibition with an apparent K_i similar to the affinity constant for FSHR-binding interaction.

In vitro *bioassays*

The inhibitory activity of the purified immunoglobulin fractions from the Ig-FSHR patients was also evaluated by bioassays. When granulosa cell preparations from 24- to 25-day-old rats where preincubated with the purified immunoglobulin fractions, these fractions significantly inhibited, in a dose-dependent manner, the production of E_2 stimulated by FSH (Fig. 5a). The figure shows two different inhibition behaviours. One (Fig. 5a, line A) corresponds to IgG fractions (from five patients) with affinity constant for the FSHR similar to that of the native hormone, and the other (Fig. 5a, line B) corresponds to IgG fractions (from five patients) that behaved as irreversible inhibitors. Purified immunoglobulin fractions from control subjects did not inhibit E_2 production.

On the other hand, the addition of immunoglobulins from Ig-FSHR patients to Leydig cell preparations did not modify LH/hCG-stimulated production of testosterone (Fig 5b). No differences were observed between the control curve and the curve corresponding to Ig-FSHR patients. Moreover, no inhibition of LH/hCG binding to Leydig cell preparations when using immunoglobulin fractions from Ig-FSHR patients was detected (Fig. 6). In addition, to further evaluate the specificity of the binding inhibition, we analysed the blocking effect of Ig-FSHR sera

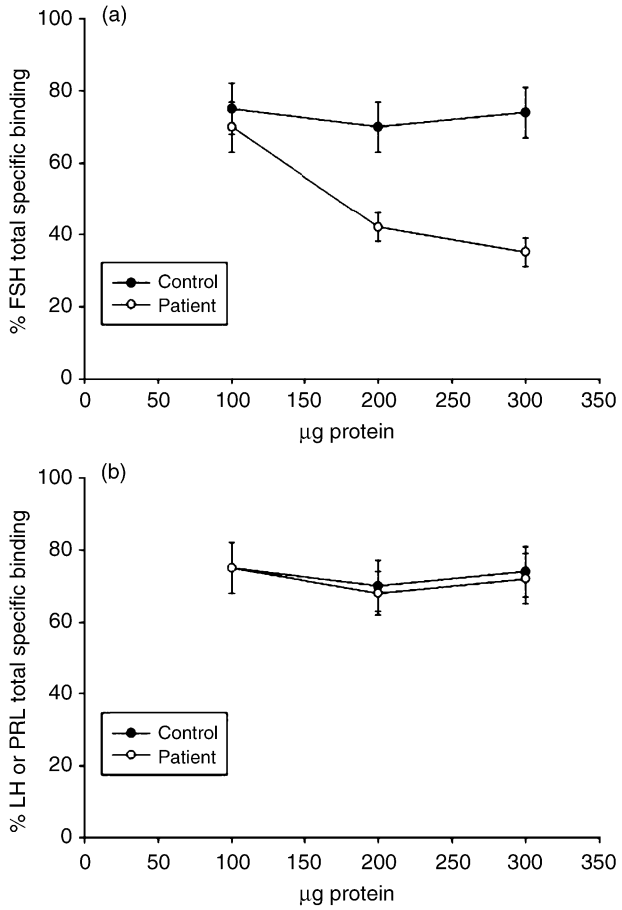

Fig. 6 Specificity of binding inhibition of purified IgG fractions from Ig-FSHR sera. (a) Effect of purified immunoglobulin fractions from controls (●) and from Ig-FSHR patients (○) on [^{125}I]FSH binding to a membrane preparation from immature rat testis. Sample illustration of all the population studied, with results corresponding to three Ig-FSHR patients and to three controls. Sera from Ig-FSHR patients inhibited [^{125}I]FSH binding to its receptor in a dose-dependent manner. Sera from the control women did not inhibit binding. (b) Effect of purified immunoglobulin fractions from controls (●) and from Ig-FSHR patients (○) on [^{125}I]LH/hCG binding to Leydig cell preparations or on [^{125}I]PRL binding to a prostate membrane preparation. Sample illustration of all the population studied, with results corresponding to three Ig-FSHR patients and to three controls. No differences were observed between curves (control versus Ig-FSHR patients). Results are expressed as the percentage of total specific binding in the absence of serum. Data are expressed as mean ± SEM.

in prostate membranes rich in PRL-receptors. None of the studied sera inhibited PRL binding to this tissue (Fig. 6).

Discussion

Receptor antibodies can either mimic the action of the hormone, like antibodies to the TSH receptor in Graves' hyperthyroidism, or altogether block the action of the corresponding hormone, like blocking antibodies to the acetylcholine receptor in myasthenia gravis. Thus, it is easily understood that receptors such as those of LH and FSH might become targets for blocking antibodies, and that such hypothetical antibodies could be a cause of ovarian refractoriness.

We had already found evidence of an inhibitor of FSH binding to rat testicular FSHR in the serum of two patients with myasthenia gravis and hypergonadotropic amenorrhoea (Escobar *et al*., 1982). We subsequently unequivocally proved that these patients had circulating IgG that blocked binding of FSH to its receptor, which might be directed towards the FSHR itself or to a receptor-related membrane domain (Chiauzzi *et al*., 1982). On this opportunity, we analysed a group of 247 patients with POF and found that 23 of them (9%) presented these antibodies.

It is noteworthy that, during the first 10 years of our investigation, patient search was specifically directed toward ROS cases, as this syndrome was by then our laboratory's target of investigation. Later on, we expanded the scope of our investigations to other POF cases, whereby a much broader spectrum of patients was derived to our laboratory from several hospitals. Thus, our tight focus on the search for ROS patients during the first 10 years eventually biased the group studied. The percentage of ROS in the later group of patients is the one which actually gets closer to the real incidence of this syndrome in population at large.

Experiments showing an interaction between antibodies from patients with gonadal failure and FSH and LH receptors, using animal tissues as receptor substrate, have already been described (Dias *et al*., 1982; Tang & Faiman, 1983; Moncayo *et al*., 1989). Subsequently, Anasti *et al*. (1995) used recombinant human gonadotropin receptors to detect immunoglobulins directed against these receptors in sera of patients with POF. They were unable to demonstrate the presence of blocking antibodies to LH or FSH receptors in any of the patients analysed. In the present study we used testis homogenates from immature rats as source of FSHRs. Although the differences between results might arise from the use of different receptor substrates, they are more likely to proceed from the differences in the patients themselves. Anasti *et al*. (1995) studied 38 patients with POF and did not detect FSHR antibodies in any. In our study, we found that only 23 out of the 247 POF patients presented these antibodies, but these Ig-FSHR patients evidenced diagnostic features which suggest that these subjects belong to a different subgroup of POF.

The 23 samples that presented Ig-FSHR had been previously diagnosed as ROS. In 20 of them, ROS diagnosis was based on the presence of numerous primordial follicles in their ovarian biopsy. The remaining three Ig-FSHR patients had been diagnosed as ROS due to an ultrasound examination that had shown ovaries above the mean size, with hyperechogenic stroma and numerous small follicular images at the periphery.

Ninety-one per cent ($n = 224$) of the patients studied resulted negative for Ig-FSHR detection. It is noteworthy that ROS diagnosis was ruled out in 48 of these negative patients, given that they had been submitted to ovarian biopsy which showed stroma with either atretic follicles or with no follicles at all. The remaining 176 negative patients had been studied by ultrasonography that revealed small ovaries (average: $2{\cdot}4 \pm 2{\cdot}1$ cm^3) with low follicular activity in some of them. We are aware that, considering the sensitivity of ultrasonography, we cannot absolutely exclude the possibility that some of them might have had some primordial follicles.

Based on the results obtained from the patients studied by biopsy, we suggest that Ig-FSHR may be present in ROS patients. The presence of antibodies directed to the FSHR itself or to a receptor-related membrane domain in these patients, is consistent with their clinical and hystopathological manifestations, and could therefore explain the failure of follicles to respond to FSH. Moreover, Arici *et al*. (2002) proposed that possible aetiologies of the ROS include antibodies against FSHR or mutations in FSHR gene.

Previous evidence of autoantibodies that block the effects of FSH in patients with ROS was provided by van Weissenbruch *et al*. (1991). Sera from 26 patients with POF were examined in order to detect IgGs that may block FSH-induced *in vitro* granulosa cell DNA synthesis via a Fuelgen cytochemical bioassay system. Ovarian growth-blocking IgGs were found in 21 out of the 26 POF cases. Among the 21 positive cases for blocking IgGs, 14 had ROS; the other seven positive cases could not be classified as ROS due to lack of specific diagnostic data. In addition, reference to a serum antibody specifically directed against the FSHR in a patient with SLE and ROS had been previously made in a case report (Anonymous, 1986). On the other hand, antibodies to FSH testis receptor were detected in serum from a male patient with primary gonadal failure, using bovine testis membranes (Dias *et al*., 1982).

Lambert *et al*. (1996) measured FSH-stimulated oestradiol production from immature rat Sertoli cells, to assess the possibility that POF may be caused by the presence of circulating FSHR blocking antibodies. Their results suggested that the presence of these antibodies is unlikely to be a major cause of POF. However, they did recognize that the small sample size ($n = 10$) they examined could have misled them to miss a low prevalence of blocking antibodies in patients with POF. Indeed, given the low incidence of ROS among POF population, we believe it is not surprising that there should not have been any ROS patients in the POF group studied.

The ovarian LH receptor does not appear to be the antigen, as we detected neither reduction in LH/hCG-stimulated production of testosterone, nor inhibition of LH/hCG binding to Leydig cell preparations, when using immunoglobulin fractions from the Ig-FSHR patients. Moreover, Austin *et al*. (1979) were also unable to detect antibodies that inhibited the binding of ^{125}I-labelled hCG to human luteal tissue, in sera from 14 POF patients. Subsequently, Wheatcroft *et al*. (1994) used an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to the LH/hCG receptor of bovine corpora lutea in a group of 45 patients with POF. In contrast with a previous report (Moncayo *et al*., 1989), they did not observe any reactivity of sera from their POF patients with fractions of bovine corpora lutea containing the LH/hCG receptor. On the other hand, we, too, were unable to find inhibition of PRL binding to membranes rich in PRL-receptors, like prostate and liver, in immunoglobulin fractions from the Ig-FSHR patients.

The relationship between ROS and myasthenia gravis in the population studied is highly interesting. Ten out of 23 ROS patients were positive for this autoimmune disorder. There are several reports informing about the prevalence of different endocrine autoimmune abnormalities in POF patients (Hoek *et al*., 1997). However, there is no reported evidence of association of autoimmune disorders with ROS syndrome, specifically.

The levels of circulating antibodies during the development of an autoimmune disorder and the time course of their appearance are potentially important to elucidate mechanisms of disease progression. In the present study, we have detected significant variations of titre of Ig-FSHR among different patients, and fluctuations along the course of the disease in the same patient. Analysis of the fluctuating course of the antibody titres in each patient might be useful to select the best moment to start treatment.

When we analysed the behaviour of the circulating inhibitor in the Ig-FSHR patients, we observed two groups. One with 'irreversible' inhibitory effect that could explain the ovarian resistance in these patients, even to the elevated endogenous bioactive gonadotropin secretion as well as to exogenous administration. Another group of patients presented the 'reversible' mechanism of inhibitory action, suggesting that the administration of a high dose of gonadotropin may, at least temporarily, reverse ovarian resistance. Further evidence of the biological implication of the inhibitory activity of the Ig-FSHR was provided by the bioassays, in which purified immunoglobulin fractions from Ig-FSHR patients inhibited the production of E_2 by granulosa cells, stimulated by FSH. The absence of inhibition of the same preparations in LH/hCG binding and in hCG-induced steroidogenesis in Leydig cell preparations, demonstrated the specificity of the FSHR-binding inhibitor. The presence of two inhibition patterns of FSH bioactivity in the *in vitro* bioassay is clearly in accordance with the two mechanisms of inhibition evidenced in binding studies. Characterization of the inhibitory behaviour of this Ig-FSHR could be of great importance to predict the response to exogenous gonadotropin administration, given that the existence of numerous ovarian follicles in the Ig-FSHR patients may allow later treatment of their infertility.

This study differs from several other published ones that have failed to demonstrate the presence of FSHR-blocking antibodies

in the serum from patients with POF (Tang & Faiman, 1983; Anasti *et al*., 1995; Lambert *et al*., 1996) probably due to differences in the patient group examined. We undertook to study a large group of POF patients ($n = 247$), thereby having higher chances of finding among them some patients with ROS, which is a very rare syndrome. Moreover, as explained above, the percentage of ROS in our group of patients does not reflect the real incidence of this syndrome in population at large. A comparable group of POF patients with a high percentage of ROS was studied by van Weissenbruch *et al*. (1991), who did find immunoglobulins that block FSH-induced granulosa cell growth *in vitro*.

We performed radioligand competition studies which are based on the ability of patient immunoglobulins to block binding of radiolabelled FSH to FSHR in rat testicular homogenates. We also included Scatchard analysis to determine the binding affinity of the Ig-FSHR, finding a remarkably high binding affinity. Even though other groups used an ELISA to detect antibodies directed toward ovarian antigens (Moncayo *et al*., 1989; Wheatcroft *et al*., 1994), we think that the competitive binding nature of the technique employed in our study, and its ability to quantify binding affinity in Scatchard analysis, constitute advantages to this approach. In addition, the present study differs from others in that natural functional steroidogenic end points, namely FSH-stimulated oestradiol production from immature rat granulosa cells, have been examined, compared with, e.g. FSH-induced granulosa DNA synthesis (van Weissenbruch *et al*., 1991) or cAMP secretion from Chinese hamster ovary cells transfected with the human FSHR (Anasti *et al*., 1995).

In conclusion, the present work suggests that Ig-FSHR may be present in ROS patients. Further research work should be done into this field, comprising a larger sample of POF subjects, in order to determine if the association found between ROS and Ig-FSHR is definitely evidenced in 100% of patients. If so, determination of the presence of Ig-FSHR could be instrumental in diagnosing the gonadotropin resistance ovary syndrome, mainly upon the basis of serological findings.

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References

Anasti, J.N., Flack, M.R., Froelich, J. & Nelson, L.M. (1995) The use of human recombinant gonadotropin receptors to search for immunoglobulin G – mediated premature ovarian failure. *Journal of Clinical Endocrinology and Metabolism*, **80**, 824–828.

Annonymous. (1986) Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises: Case 46–1986. *New England Journal of Medicine*, **315**, 1336–1343.

Arici, A., Matalliotakis, I.M., Koumantakis, G.E., Goumenou, A.G., Neonaki, M.A. & Koumantakis, E.E. (2002) Diagnostic role of inhibin B in resistant ovary syndrome associated with secondary amenorrhea. *Fertility and Sterility*, **78**, 1324–1326.

Austin, G.E., Coulam, C.B. & Ryan, R.J. (1979) A search for antibodies to luteinizing hormone receptors in premature ovarian failure. *Mayo Clinic Proceedings*, **54**, 394–400.

Barañao, J.L., Bley, M.A., Batista, F.D. & Glikin, G.C. (1991) A DNA topoisomerase I inhibitor blocks the differentiation of rat granulosa cells induced by follicle-stimulating hormone. *Biochemical Journal*, **277**, 557–560.

Best-Belpomme, M. & Dessen, P. (1973) Inhibition compétitive. Relations linèares nouvelles et gènerales. *Biochimie*, **55**, 11–16.

Board, J.A., Redwine, F.O., Moncure, C.W., Frable, W.J. & Taylor, J.R. (1979) Identification of differing etiologies of clinically diagnosed premature menopause. *American Journal of Obstetrics and Gynecology*, **134**, 936–941.

Charreau, E.H., Attramadal, A., Torjesen, P.A., Calandra, R., Purvis, K. & Hansson, V. (1977) Androgen stimulation of prolactin receptors in rat prostate. *Molecular and Cellular Endocrinology*, **7**, 1–7.

Charreau, E.H., Calvo, J.C., Tesone, M., de Souza Valle, L.B. & Baranao, J.L. (1978) Insulin regulation of Leydig cell luteinizing hormone receptors. *Journal of Biological Chemistry*, **253**, 2504–2506.

Chiauzzi, V., Cigorraga, S., Escobar, M.E., Rivarola, M.A. & Charreau, E.H. (1982) Inhibition of follicle-stimulating hormone receptor binding by circulating immunoglobulins. *Journal of Clinical Endocrinology and Metabolism*, **54**, 1221–1228.

Coulam, C.B. & Ryan, R.J. (1979) Premature menopause. I. Etiology. *American Journal of Obstetrics and Gynecology*, **133**, 639–643.

Coulam, C.B., Adamson, S.C. & Annegers, J. (1986) Incidence of premature ovarian failure. *Obstetrics and Gynecology*, **67**, 604–606.

Dias, J.A., Gates, S.A. & Reichert, L.E. Jr (1982) Evidence for the presence of follicle-stimulating hormone receptor antibody in human serum. *Fertility and Sterility*, **38**, 330–338.

Elder, M., Maclaren, N. & Riley, W. (1981) Gonadal autoantibodies in patients with hypogonadism and/or Addison's disease. *Journal of Clinical Endocrinology and Metabolism*, **52**, 1137–1142.

Escobar, M.E., Cigorraga, S.B., Chiauzzi, V.A., Charreau, E.H. & Rivarola, M.A. (1982) Development of the gonadotrophic resistant ovary syndrome in myasthenia gravis: suggestion of similar autoimmune mechanisms. *Acta Endocrinologica*, **99**, 431–436.

Flier, J.S., Kahn, C.R., Roth, J. & Bar, R.S. (1975) Antibodies that impair insulin receptor binding in an unusual diabetic syndrome with severe insulin resistance. *Science*, **190**, 63–65.

Fox, H. (1992) The pathology of premature ovarian failure. *Journal of Pathology*, **167**, 357–363.

Hoek, A., Schoemaker, J. & Drexhage, H.A. (1997) Premature ovarian failure and ovarian autoimmunity. *Endocrine Reviews*, **18**, 107–134.

Jones, G.S. & de Moraes-Ruehsen, M. (1969) A new syndrome of amenorrhea in association with hypergonadotropism and apparently normal ovarian follicular apparatus. *American Journal of Obstetrics and Gynecology*, **104**, 597–600.

Kamp, P., Platz, P. & Nerup, J. (1974) 'Steroid-cell' antibody in endocrine diseases. *Acta Endocrinologica*, **76**, 729–740.

LaBarbera, A.R., Miller, M.M., Ober, C. & Rebar, R.W. (1988) Autoimmune etiology in premature ovarian failure. *American Journal of Reproductive Immunology and Microbiology*, **16**, 115–122.

Lambert, A., Weetman, A.P., McLoughlin, J., Wardle, C., Sunderland, J., Wheatcroft, N., Anobile, C. & Robertson, W.R. (1996) A search for immunoglobulins inhibiting gonadal cell steroidogenesis in premature ovarian failure. *Human Reproduction*, **11**, 1871–1876.

Manley, S.W., Bourke, J.R. & Hawker, R.W. (1974) The thyrotrophin receptor in guinea-pig thyroid homogenate: Interaction with the long-acting thyroid stimulator. *Journal of Endocrinology*, **61**, 437–445.

Mendelson, C., Dufau, M.L. & Catt, K.J. (1975) Gonadotropin binding and stimulation of cyclic adenosine 3′:5′-monophosphate and testosterone production in isolated Leydig cells. *Journal of Biological Chemistry*, **250**, 8818–8823.

Moncayo, H., Moncayo, R., Bentz, R., Wolf, A. & Lauritzen, C. (1989) Ovarian failure and autoimmunity. Detection of autoantibodies directed against both the unoccupied luteinizing hormone/human chorionic gonadotropin receptor and the hormone receptor complex of bovine corpus luteum. *Journal of Clinical Investigation*, **84**, 1857–1865.

Patrick, J. & Lindstrom, J. (1973) Autoimmune response to acetylcholine receptor. *Science*, **180**, 871–872.

Salvi, M., Fukazawa, H., Bernard, N., Hiromatsu, Y., How, J. & Wall, J.R. (1988) Role of autoantibodies in the pathogenesis and association of endocrine autoimmune disorders. *Endocrine Reviews*, **9**, 450–466.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Annals of the New York Academy of Sciences*, **51**, 660–672.

Tang, V.W. & Faiman, C. (1983) Premature ovarian failure: a search for circulating factors against gonadotropin receptors. *American Journal of Obstetrics and Gynecology*, **146**, 816–821.

Tesone, M., Biella de Souza Valle, L., Foglia, V.G. & Charreau, E.H. (1976) Endocrine function of the testis in streptozotocin diabetic rats. *Acta Physiologica Latino Americana*, **26**, 387–394.

Vincent, A. & Newsom-Davis, J. (1985) Acetylcholine receptor antibody as a diagnostic test for myasthenia gravis: results in 153 validated cases and 2967 diagnostic assays. *Journal of Neurology, Neurosurgery, and Psychiatry*, **48**, 1246–1252.

van Weissenbruch, M.M., Hoek, A., van Vliet-Bleeker, I., Schoemaker, J. & Drexhage, H. (1991) Evidence for existence of immunoglobulins that block ovarian granulosa cell growth *in vitro*. A putative role in resistant ovary syndrome? *Journal of Clinical Endocrinology and Metababolism*, **73**, 360–367.

Wheatcroft, N.J., Toogood, A.A., Li, T.C., Cooke, I.D. & Weetman, A.P. (1994) Detection of antibodies to ovarian antigens in women with premature ovarian failure. *Clinical and Experimental Immunology*, **96**, 122–128.

Yen, S.S., Tsai, C.C., Vandenberg, G. & Rebar, R. (1972) Gonadotropin dynamics in patients with gonadal dysgenesis: a model for the study of gonadotropin regulation. *Journal of Clinical Endocrinology and Metabolism*, **35**, 897–904.