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

H. Moncayo, R. Moncayo,* R. Benz, A. Wolf, and Ch. Lauritzen

Department of Obstetrics and Gynecology, and *Department of Internal Medicine, University of Ulm, D-7900 Ulm, Federal Republic of Germany

Abstract

We developed an ELISA system for the detection of human anti-ovarian antibodies. Bovine corpora lutea were extracted in PBS (pH 7.2) and fractionated by ultracentrifugation. Both the soluble fraction obtained after 80,000 g (S80) and the Triton-extracted membrane fraction (ST288) were used as antigens. Additionally, the luteinizing hormone (LH)/human chorionic gonadotropin (hCG) receptor was isolated by affinity chromatography (wheat germ agglutinin and LH-Sepharose) and also used as an antigen.

In 7 of 14 patients with primary sterility and endometriosis a positive reaction was observed. Similarly, 6 of 16 patients with secondary sterility and endometriosis were also positive. Patients being stimulated for in vitro fertilization and presenting either primary or secondary sterility were positive in 5 of 22 and 6 of 16 cases, respectively. In the S80 test 41 of 60 sera presented IgG₂ antibodies, whereas in the ST288 test 38 of 60 belonged to the IgG₁ subclass. Kappa and lambda chains were equally distributed.

Some patients could recognize the unoccupied LH/hCG receptor as an antigen, while others recognized only the complex formed by the hormone plus the hormone receptor.

The S80 and ST288 antigens were isolated by affinity chromatography. Gel permeation of the purified antigens revealed in each case the presence of an antigen complex. The apparent molecular weight was between 2,000 and 36,000 D. Cross-reactivity studies using affinity-purified antibodies demonstrated an antigenic relationship of the membrane, soluble, and extractable fractions. NAc-(beta-1 → 4)-D-glucosaminide and -D-galactopyranoside were the main terminal glycosides.

Introduction

Antibodies directed against ovarian tissue have been described by several authors. The first studies using indirect immunofluorescence (IFL)¹ were carried out on patients who had either an adrenal insufficiency or a pluriglandular autoimmune deficiency coupled to either premature ovarian failure or infertility

(1–6). These ovarian antibodies (Ov-Ab) were considered to be part of an immune reaction involving steroid-producing cells. The purely morphological data arising from IFL have allowed recognition of different staining patterns, including Leydig cells, dispersed single cells in the ovarian interstitium, ovarian theca cells, corpus luteum, granulosa cells, and oocytes (1–3, 7). In a follow-up study of patients with autoimmune polyglandular disease the appearance of Ov-Ab was found to be associated with a loss of ovarian function (6). Despite these first conceptual interpretations of the results, i.e., Ov-Ab being exclusively associated with polyendocrinopathies, a lack of relationship to either adrenal or pluriglandular insufficiency has also been observed (8–11). Additional clinical settings where Ov-Ab can be observed are premature menopause, endometriosis, and systemic lupus erythematosus (7, 10, 12, 13).

The institution of an immunosuppressive treatment in some cases of autoimmune ovarian failure (7, 14–17) has led to a normalization of the menstrual cycle and even to pregnancy, thus revealing a functional relationship of the antibody finding to infertility. Theoretically, the hormone receptors for follicle-stimulating hormone (FSH) and luteinizing hormone (LH) could be the target in cases of autoimmune infertility. Austin et al. (11) and Tang and Faiman (18) failed to demonstrate this hypothesis. However, antibodies that interfered with the binding of FSH to its receptor were referred to in a recent case discussion of a patient with lupus erythematosus associated with premature ovarian failure (19). Unfortunately, no precise data were provided on the hormone-binding assay. Similarly, antibodies directed against the FSH-hormone receptor were described in one case of myasthenia gravis (20). Recent histopathological descriptions of autoimmune oophoritis include cellular infiltrates with plasma cells and lymphocytes affecting developing, cystic, and atretic follicles, and even surrounding nerve fibers (21, 22). Ovary-bound antibodies have also been detected in cases of infertility (23).

Although all these evidences suggest that the ovary can be considered a target organ for autoimmune processes within the field of the immunology of reproduction, recent reviews on this topic have practically omitted the ovary as a possible antigen (24–26).

The aim of this study was to use different techniques for approaching the question of whether patients with infertility can present Ov-Ab, and to attempt to isolate and characterize the putative antigens. Departing from the conventional approach of IFL, an ELISA procedure was used to detect and characterize the human antibodies.

Methods

Subjects

A total of 170 persons were included in this study. They belonged to 1 of the 10 following study groups. The age range was comparable in all groups (18–38 yr).

Address correspondence to Dr. Helga Moncayo, University of Innsbruck, Department of Obstetrics and Gynecology, Anichstrasse 35, A-6020 Innsbruck, Austria.

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1. Abbreviations used in this paper: hCG, human chorionic gonadotropin; IFL, indirect immunofluorescence; IVF, in vitro fertilization; Ov-Ab, ovarian antibodies.

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Controls. 30 healthy persons, 20 women and 10 men (hospital and laboratory personnel) without any known disease, served as the control group. Additionally, 22 normal adult blood donors were also investigated in the IgG S80 test.

Postpartum thyroiditis. 20 women presenting with postpartum thyroiditis were studied 3–4 wk after delivery. All of them presented with elevated titers of thyroid antibodies.

Hashimoto thyroiditis. 8 female patients with Hashimoto thyroiditis and presenting with elevated titers of thyroid antibodies were studied.

Addison's disease. A total of 15 patients with Addison's disease were subdivided into two groups according to the results of the IFL for the determination of anti-adrenal antibodies. 8 patients had a positive IFL result and 7 had a negative result.

Polyendocrinopathy. 7 patients presenting with polyendocrinopathy were included also. The main characteristics included diabetes mellitus, adrenal insufficiency, thyroid disease, and hemolytic anemia. All of them were positive for adrenal antibodies on IFL.

Patients under treatment for in vitro fertilization (IVF). A total of 38 patients were included. 22 presented with primary sterility, while 16 had secondary sterility. All patients were normogonadotropic and had no additional endocrine disease.

Patients with endometriosis. 30 patients with endometriosis were studied. The diagnosis was confirmed by pelviscopy. 14 patients sought medical assistance for primary sterility and 16 for secondary sterility. All patients were normogonadotropic and were treated with luteinizing hormone–releasing hormone analog.

Buffer systems for the ELISA procedures

Extraction and coating buffer contained 23.279 g $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$, 3.45 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and 81.816 g NaCl per 10 liters, adjusted to pH 7.2 (PBS). Dilution buffer was the same coating buffer to which 0.05% Tween (vol/vol) had been added (PBS-T). Substrate buffer for alkaline phosphatase contained 6.0 g $\text{Na}_2\text{CO}_3 \cdot 3 \cdot 10 \text{H}_2\text{O}$, 2.436 g NaHCO_3 , and 0.203 g $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ per liter, adjusted to pH 9.8. Before use, phosphatase substrate (Sigma Chemie GmbH, Munich, FRG), 1 mg/ml, was added. Substrate buffer for peroxidase was made by mixing 3.5 ml of 1 M citric acid with 6.5 ml of 1 M sodium citrate and adding distilled water up to a volume of 100 ml. Before use, 200 mg orthophenylenediamin and 400 μl of 30% H_2O_2 /100 ml were added and mixed thoroughly.

Ovary extraction

Bovine ovaries were obtained at the local abattoir and transported in ice-cold PBS to the laboratory. All further steps were carried out in a cold room at 4°C. After carefully excising the connective and fibrous tissue the corpora lutea were dissected and subjected to homogenization using a mechanical device (Waring blender). Approximately 4 ml of cold extraction buffer was added per g tissue. The resulting homogenate was filtered through two plies of gauze and then centrifuged at 3,000 g for 30 min. The 3,000 g supernatant was then centrifuged at 20,000, 80,000, and 288,000 g for 60 min each using a swing-out rotor (SW27; Beckman, Munich, FRG). The 18,000 g pellet was solubilized using 1% Triton in PBS during 60 min at room temperature followed by centrifugation at 288,000 g. Table I summarizes the extraction procedure and describes the abbreviations used for the different fractions. Protein concentration was measured using a modification of the Bradford method (27) where 10 μl of the sample was allowed to react with 150 μl of the brilliant blue G reagent. The OD was measured using an automatic photometer (MR600; Dynatech, Denkendorf, FRG). BSA in PBS was the standard.

Isolation of the LH/hCG receptor

The protocol from Kusuda and Dufau (28) was reproduced using lectin affinity chromatography with wheat germ agglutinin and affinity chromatography with an LH-Sepharose gel. The purified LH preparation was obtained from the National Institutes of Health. Two receptor

Table I. Flowsheet for the Preparation of Bovine Corpus Luteum Fractions

Transport fresh bovine ovaries on ice-cold PBS to the laboratory. Dissect both fatty and connective tissue, mince the corpora lutea finely, and wash three times with PBS. Add 4 ml PBS/g ovary and homogenize using a blender (Waring Products Div., New Hartford, CT). Filter the raw homogenate through two plies of gauze.

Centrifuge at 150 g for 30 min at 4°C.

Discard the pellet P1 and save the supernatant S1.

Centrifuge S1 at 3,000 g for 30 min at 4°C.

Discard the pellet P2 and save the supernatant S2.

Centrifuge S2 at 20,000 g for 30 min at 4°C.

P3 pellet (membrane fraction)	S3 supernatant (soluble fraction)
Wash the pellet three times with PBS. Centrifuge at 20,000 g for 30 min at 4°C.	Aspirate the clear supernatant discarding the upper fatty phase.
Redissolve P3 in PBS with 1% Triton X-100. Incubate at room temperature for 30 min.	Centrifuge at 80,000 g for 60 min at 4°C. Save both P4 and S4.
Centrifuge at 288,000 g for 120 min at 4°C.	Centrifuge S4 at 288,000 g for 120 min at 4°C.
Save P3T and S3T.	Save S5.

P1, 150 g pellet; P2, 3,000 g pellet; S2, 2,000 g supernatant; P3, 18,000 g pellet; S3, 18,000 g supernatant; P3T, 288,000 g pellet after solubilization with 1% Triton-PBS; ST, 288,000 g supernatant after Triton; S4 or S80, supernatant after 80,000 g; S5 or S288, supernatant after 288,000 g.

fractions were eluted from the LH gel using glycine (50 mM, pH 4.5) and acetic acid (25 mM, pH 4.0). The eluates were immediately neutralized using a 1 M Tris buffer and adjusted to pH 7.2.

ELISA procedures

The organ extracts were diluted to a concentration of 5 μg protein/ml by adding the required volume of coating buffer. The microtiter plates (M129B Immulon II flat bottom; Dynatech) were coated with the respective extracts by adding 200 μl /well and incubating overnight at room temperature (20°C). After coating, the plates were washed three times using PBS-T. The sera were initially tested in the following dilutions: 1:10, 1:40, 1:160, 1:640, 1:2,560, 1:10,240, 1:40,960, and 1:163,840 with 100 μl /well. The dilution curve was done in singles. The dilution buffer was PBS-T. The plates were incubated for 60 min at 37°C and then washed three times with PBS-T. Bound human antibodies were detected by adding 100 μl of affinity chromatography–purified goat F(ab')₂ anti-human IgG and IgM, labeled with alkaline phosphatase (anti-IgG 2,490, anti-IgM 2,492; Tago Inc., Burlingame, CA) obtained from MEDAC, Hamburg, FRG. The conjugates were diluted with PBS-T according to the instructions of the manufacturer (1:4,500 and 1:9,000, respectively). After incubating the plates at 37°C for 60 min they were washed three times with PBS-T. 100 μl of phosphatase substrate buffer was then added and incubated for 30 min. The OD was measured with an automatic photometer (MR600, Wavelength 405 nm; Dynatech). The results were transferred on-line to an Apple II computer and analyzed.

Detection of antibodies directed against the hormone receptor

38 control samples and 35 patients were tested in an indirect ELISA that had the purified receptor as the antigen at a concentration of 500 ng/well. Using a commercially available human chorionic gonadotropin (hCG) preparation (Primogonyl-5000; Schering, Berlin, FRG) the receptor was exposed to increasing concentrations of the hormone. The hCG was diluted with PBS-T to achieve the following final concentrations: 0, 1, 10, and 100 U/well. The hormone preparation was incubated at 37°C for 2 h. Afterwards the plates were washed and the diluted sera (1:50 with PBS-T) were added and incubated as indicated above. The OD read with no hormone added was taken as the starting 100% binding for each serum tested. An individual curve was constructed for each of the patients. A control inhibition procedure was carried out with bovine thyrotropin-stimulating hormone (TSH) (Organon, Munich, FRG).

Characterization of IgG subclasses

60 serum samples were reevaluated to characterize the IgG subclasses as well as the kappa and lambda light chains of the antibodies. Plates were coated with the S80 and the ST288 antigen fractions (5 µg protein/ml, 120 µl/well, overnight at room temperature). One predilution step 1:50 was done using 1.4 ml Macrowells (Skatron, Norderstedt, FRG). Thus, by using the same dilution of each sample in the different tests, variability was minimized. Specific mouse MABs directed against the four IgG classes and the kappa and lambda chains were obtained from Unipath (Cambridge, UK). Each conjugate was diluted 1:1,000 with PBS-T. The clones used were 8a4 for total IgG, NL16 for IgG₁, HP6014 for IgG₂, ZG4 for IgG₃, RJ4 for IgG₄, C4 for kappa, and 6el for the lambda chains occupying rows A–G of the microtiter plate, respectively, 100 µl/well. Row H contained no MAB and served as the blank value for each test. The plates were incubated at 37°C for 60 min and then washed three times with PBS-T. An affinity chromatography-purified, peroxidase-labeled, anti-mouse antibody (Tago, Inc.) was diluted 1:2,500 with PBS-T, and 100 µl was added to each well and incubated for 60 min at 37°C. After washing three times with PBS-T, peroxidase substrate buffer was finally added and incubated for 5 min in the dark. The color reaction was stopped by the addition of 100 µl 1 N HCl. The OD was measured at 490 nm.

ELISA data analysis

Reference values were calculated by integration of the area under the curve. The mean value plus 3 SD was taken as the upper limit for each test. The area under the curve obtained from the unknowns was divided by the value of the negative control sample, which had been arbitrarily assigned 100%. A result was called positive when it exceeded the mean plus 3 SD of the corresponding reference value. Both a negative and a positive quality control sample were measured in each test. In the assay for IgG subclasses the values obtained for each of the subclasses were added to give a total IgG score. The individual subclasses were expressed as a percent thereof. The data for kappa and lambda chains were also analyzed as a percentage of the total (100% = kappa + lambda).

Antibody isolation by ELISA-AFFIN

A modified ELISA test was used to obtain isolated specific antibodies from sera that showed a positive reaction. In a separate assay the microtiter plates were coated with the following antigens: P3, ST288, and S288. 250 µl of an antigen solution adjusted to a protein concentration of 50 µg/ml was added to each well and allowed to coat overnight at room temperature. After washing the plates three times with PBS-T, 100 µl of serum was added to four wells and further diluted 1:2.5 by adding 150 µl of PBS-T. After the serum incubation (60 min, 37°C) the plates were washed three times with PBS-T and the bound antibodies were eluted using a 2 M NaCl solution that had been previously brought to 37°C. The plates were incubated for 60 min at 37°C with the NaCl solution. Afterwards the extract corresponding to each

patient was collected and desalted on a G-25 Sephadex (Pharmacia, Freiburg, FRG) column that had been preequilibrated with PBS. The concentration of IgG, IgA, and IgM in both the original sample and the resulting extract was measured using a routine nephelometry test (Behring, Marburg, FRG). After the antibody extraction, the ELISA procedure was completed as usual to check for any residual bound antibodies.

Cross-reactivity of the ovarian fractions

The ELISA-AFFIN procedure (see above) was used to obtain specific antibodies that react with each of the ovarian fractions, namely, P3, ST288, and S288. In a crossover test each individual extract was tested with the other fractions. Incubation conditions were as described above for the routine test except for the dilution steps, which were 1:10 and 1:50. Rows G and H contained no antigen as they were used to evaluate the nonspecific binding. Only IgG antibodies were detected in this experiment using the corresponding conjugate.

Cross-reactivity with thyroid and liver

A series of 10 positive sera were preincubated on plates containing 5 µg protein/ml in PBS of thyroid and liver extracts. For these experiments the soluble fraction obtained after homogenization and centrifugation at 80,000 g was used. After preincubating 60 min at 37°C the sera were transferred to microtiter plates containing the ovarian fractions and the ELISA was conducted as usual. In each experiment the preincubation control was a separate plate that had been incubated with PBS buffer alone without any antigen.

Chromatographical studies

All procedures were done using the fast protein liquid chromatography system from Pharmacia (Freiburg, FRG). The ovarian antigen fractions were characterized by means of gel permeation chromatography using a Superose 12 column that had been preequilibrated with PBS. Chromatofocusing in a pH range of 6.3–4 was carried out according to the instructions of the manufacturer with the MONO-P column. Each of the fractions obtained were also tested in the ELISA system. For the affinity chromatography procedures the antigens and antibodies were coupled to cyanbromide-activated Sepharose-4B according to the instructions of the manufacturer (36). Elution off the affinity columns was achieved using 0.2 M glycine, pH 2.5. The eluted fractions were immediately neutralized by adding a few drops of 1 M Tris-HCl buffer to achieve a pH of 7.2. Thereafter the fractions were desalted using a G-25 column.

ELISA with the chromatography fractions

The fractions obtained after gel permeation and chromatofocusing were used directly to coat microtiter plates. Patient's serum was diluted 1:100 with PBS-T and allowed to react with the individual fractions. Each plate contained 10 different fractions and 2 blank controls. OD values exceeding the values of the blank wells by 300% were called positive.

Determination of the terminal glycoside residues of the antigens

A peroxidase-labeled lectin kit containing Con A, *Dolichous biflorus*, *Griffonia simplicifolia*, *Maclura pomifera*, *Arachis hypogaea*, soybean, *Ulex europaeus*, and wheat germ agglutinin was used to determine the terminal glycoside residues (lectin kit HLK-001; E. Y. Laboratories Inc., San Mateo, CA, obtained through MEDAC, Hamburg, FRG). The 80,000 g supernatant fraction, the Triton-extracted 20,000 g pellet (288,000 g supernatant), and the pellet remaining after the Triton extraction as well as the fractions obtained after gel permeation chromatography of S80 and ST288 were studied. Microtiter plates were coated with a solution containing 5 µg protein/ml of the S80, PT288, and ST288 fractions in separate assays, whereas the resulting chromatography products (i.e., each 2-ml fraction) were used directly for coating overnight at room temperature. Each lectin was diluted

1:1,000 with PBS-T. 100 μ l were added to each well and incubated at 37°C for 60 min. After washing the plates three times with PBS-T, peroxidase substrate buffer with 1 mg orthophenyldiamin/ml was added (100 μ l) and incubated for 5 min. The color reaction was stopped with 100 μ l of 1 N HCl. The OD was read at 490 nm.

Results

ELISA system. Antigen coating, conjugate dilution, and substrate were used as stated in Methods. Both of the soluble fractions, S80 and S288, were equally reactive as antigens. The optimal dilution curves of the serum were 1:50, 1:200, 1:800, and 1:3,200, 150 μ l of each per well. The color reaction was linear between 15 and 90 min. The color reaction was not stopped; the plates were always read 30 min after adding the substrate. Based on the initial negative reference sample used throughout, the mean values of the area under the curve expressed as the OD $\times$ 1,000 for the negative and positive quality control samples were: (a) S80 test (soluble antigen), negative sample 512%, positive sample 2,484% for IgG and 318 and 1,413%, respectively, for IgM; and (b) ST288 test (extractable antigen), negative sample 482%, positive sample 1,524% for IgG and 333 and 1,298%, respectively, for IgM. Tests that showed great deviations from the above-mentioned quality control means were rejected and repeated. The coefficient of variation between assays was 12–15%.

Controls. The mean OD values and the SD for both IgG and IgM antibodies were 123 $\pm$ 39 and 76 $\pm$ 17% for S80 and, 99 $\pm$ 29 and 85 $\pm$ 25% for ST288, respectively. The upper

limits were defined as the mean of the control group plus 3 SD: 240 and 200% for IgG and IgM, respectively, in the S80 test, and 215 and 200% for IgG and IgM, respectively, in the ST288 test. In the LH/hCG receptor test the mean value for IgG antibodies was 141 $\pm$ 45% and the upper limit was 280%. Samples exceeding these values were called positive.

While all of the study groups could be tested against the soluble and the extractable ovarian antigens (Figs. 1 and 2; Table II), only 38 controls and 35 patients with sterility could be tested against the isolated LH/hCG receptor (Fig. 3). The main limitation in this study was the small amount of receptor protein available. However, the sera tested included representative examples for normal and elevated binding values (similar to Figs. 1 and 2).

Patients with thyroiditis. Both the patients with postpartum thyroiditis and those with Hashimoto thyroiditis presented normal values in 26 of 28 cases (93%). The two patients with postpartum thyroiditis who were positive on the Ov-Ab test were also positive for endometrial antibodies (data not shown).

Patients with Addison's disease. Only 2 of 8 patients who presented a positive reaction against adrenal tissue on IFL showed a positive reaction against the ovarian fractions. All patients with a negative result on the IFL were also negative for Ov-Ab.

Patients with polyendocrinopathy. Five of the seven patients with polyendocrinopathy were positive for Ov-Ab.

Patients with primary and secondary sterility. Altogether, 24 of 68 patients with primary and secondary sterility showed

Figure 1. IgG Ov-Ab directed against the soluble corpus luteum antigen S80. All of the observed values for the 10 study groups are included in a dot plot. The filled signs represent those cases that lay within the defined normal range. The study groups were: A, controls, $n = 52$; B, postpartum thyroiditis, $n = 20$; C, Hashimoto thyroiditis, $n = 8$; D, Addison's disease with positive results for adrenal antibodies on indirect immunofluorescence, $n = 8$; E, Addison's disease with negative results for adrenal antibodies on IFL, $n = 7$; F, polyendocrinopathy patients, $n = 7$; G, patients with primary sterility being treated for IVF, $n = 22$; H, patients with secondary sterility being treated for IVF, $n = 16$; I, patients with primary sterility and endometriosis, $n = 14$; J, patients with secondary sterility and endometriosis, $n = 16$.

Figure 2. IgG Ov-Ab directed against the extractable corpus luteum antigen ST288. All of the observed values for the 10 study groups are included in a dot plot. The signs and group codes are the same as in Fig. 1.

Table II. Summary of the Results of IgG Ov-Ab in the S80 Test

Group	IgG Ov-Ab (mean + SD)					
	Negative subgroup < 240%			Positive subgroup > 240%		
	n	Mean	SD	n	Mean	SD
Normals	52	123	39	—	—	—
Postpartum thyroiditis	18	137	40	2	497	218*
Hashimoto thyroiditis	8	148	38	—	—	—
Addison's disease (with reference to IFL adrenal antibody results)						
Positive IFL [‡]	6	125	34	2	308	18 [§]
Negative IFL [‡]	7	122	56	—	—	—
Polyendocrinopathy						
Positive IFL [‡]	2	188	25	5	333	150 [§]
IVF patients						
Primary sterility	17	94	40	5	422	140 [§]
Secondary sterility	10	125	28	6	428	124 [§]
Patients with endometriosis						
Primary sterility	7	130	22	7	352	157 [§]
Secondary sterility	10	132	25	6	386	77 [§]

The results of the ELISA test using the soluble S80 antigen are expressed as a relative binding index in percent taking as a reference an upper range of 240% which corresponds to the mean plus 3 SD of the values obtained from the normals. Corresponding dot plot is shown in Fig. 1.

* $P < 0.05$.

[‡] The patients were subclassified according to the results of the conventional IFL for the detection of adrenal antibodies of IgG class.

[§] $P < 0.01$ in unpaired t test.

^{||} These patients were receiving clomiphene and hCG/hMG to induce ovulation.

positive results. The majority of positive results were IgG antibodies in 90% of the cases. In the group of patients being treated for IVF 5 of 22 cases with primary sterility were positive, while 6 of 16 cases with secondary sterility were also positive. Since these patients were constantly being controlled for IVF they were investigated two to three times in the ELISA system. All patients with positive results remained positive during the observation period of 3–4 mo. In the second group of patients presenting with endometriosis, 7 of 14 cases with primary sterility were positive, as were 6 of 16 with secondary sterility.

Antibodies directed against the LH/hCG receptor. 35 patients with sterility could be tested. Inclusion of the other con-

Figure 3. IgG directed against the isolated LH/hCG hormone receptor. Group A includes the normal controls, group B those patients with sterility presenting with normal values in the test, and group C, sterility patients presenting elevated values.

trol groups was not possible due to the limited amount of isolated antigen. 24 patients presented normal values when tested against the unoccupied receptor (183 ± 40 , mean $\pm$ SD), while 11 patients presented a positive result for IgG antibodies in this assay. The range of positive values was 310–750%; the mean and SD were $460 \pm 146\%$ ($P < 0.01$, unpaired t test; Fig. 3). Five patients reacted with the occupied receptor after saturating with hCG. At the same time this presaturation step led to a decreased binding index in other sera, thus two general binding patterns were discernable: (a) binding to the unoccupied receptor which can be displaced by hCG leading to a decrease of the initial values, and (b) binding to the occupied receptor with increasing values when hCG preincubation in increasing dosages has occurred. These patterns are shown in Fig. 4. Curves A and C represent the cases where the binding intensity was found to decrease or increase continuously, respectively, as a function of the added amount of hCG in the preincubation step (0–100 U/well). Curves B and D represent those cases that showed a plateau at the maximal hCG concentration of 10–100 U/well. Inhibition with TSH affected the antibody binding in only one case showing a decrease.

Ig subclasses. The observed percentages of the positive samples are reported according to the highest values observed for each of the IgG subclasses. When a tie was found, a positive result was counted for each subclass, therefore exceeding the number of tested sera. The number of positive observations for each of the IgG subclasses according to the antigen tested were as follows: for S80, IgG₁ 10, IgG₂ 41, IgG₃ 0, and IgG₄ 12; for ST288, IgG₁ 38, IgG₂ 18, IgG₃ 1, and IgG₄ 9. Thus, a significant association was noted for IgG₂ antibodies and the soluble antigen and for IgG₁ antibodies and the extractable antigen. With few exceptions the kappa and lambda distribution was 40–60% kappa and 60–40% lambda.

Antigen characterization using gel permeation and chromatofocusing. Fig. 5 shows the characteristic profiles of the S80 and ST288 fractions after gel permeation. Fig. 6 shows the chromatofocusing profile of ST288. The apparent molecular weights and the proportion of each peak area in the whole fraction is shown in Table III together with the corresponding terminal sugar residues. In a second set of experiments the Triton X-100 extract, the pellet remaining after Triton treatment, and the soluble fraction were analyzed. A similar pat-

tern to the one described in Table III was observed in all three fractions, thus revealing that the soluble antigen fraction, S80, also contains glycosylated proteins.

ELISA with the chromatography fractions. When the sera were tested against the S80 fractions obtained by gel permeation, binding was seen in two broad molecular weight regions; namely, between 100,000 and 300,000 D and between 9,200 and 2,500 D. With the extractable fraction ST288 a similar binding pattern was observed whereby the binding regions included 470,000 and 47,000 D and < 500 D.

After chromatofocusing of the ST288 fraction, serum binding to both main peaks was observed. Fig. 6 shows the binding of human antibodies to the individual fractions. In preliminary studies binding of radioactive labeled LH was shown to occur with the peak with a pI of 6.0 (data not shown). Thus it is evident that components of this fraction, which are probably not the hormone receptor, can also be recognized as antigens.

Isolation of the antibodies by ELISA-AFFIN and by affinity chromatography. Anti-Ov-Ab were isolated from a pool of sera with Ov-Ab. Although the ELISA-AFFIN method was useful for the isolation of antibodies, its low capacity ($< 100 \mu\text{g}$ IgG) was the major drawback for large scale experiments. Approximately 10% of the original OD values were still found bound after eluting the antibodies from the plates. The amount of isolated antibodies was 1 μg out of 10–100 mg total IgG. Human Ov-Ab isolated by affinity chromatography were used for the antigen isolation experiments with 1 mg of human anti-ovarian IgG.

Cross-reactivity studies. Antibodies were isolated using three different raw antigen fractions, P3, S80, and ST288, in separate experiments according to the ELISA-AFFIN method. When tested with the other fractions, 9 of 10 individual sera reacted positively. In the experiments that used thyroid and liver extracts bound to a solid phase for preincubating the diluted sera, only 2 of 10 cases showed an inhibition effect with each organ. The reduction of the OD was 30–70% compared with the uninhibited values.

Discussion

Since the original report of Irvine et al. (1) the detection of Ov-Ab has been almost exclusively associated with a group of patients with Addison's disease or polyendocrine insufficiency (2–6). These associations are not exclusive since many other authors have found no relation to any autoimmune endocrine deficiencies (6, 8, 11). Besides the absorption studies done by Irvine et al. (1) using saline organ extracts and those of de Moraes-Ruehsen et al. (3) and Elder et al. (5), and the use of radiolabeled ovarian extracts by Coulam and Ryan (14), there have been very few attempts to deliver more precise data on the nature of the putative autoantigens. These experiments, however, suggested that the antigen(s) must be contained in the extracts used. To overcome the methodological drawback connected with the use of IFL we have successfully used different methods for antibody detection, antibody isolation, and antigen purification. The general applicability of this approach has been recently demonstrated by us with the successful isolation of antigens relevant to other autoimmune diseases (30, 31).

To avoid the drawbacks of IFL such as extremely heterogeneous species (human, rat, monkey, and rabbit), and different functional ovarian stages (virgin, mature, pregnant, pseudo-

Figure 4. Binding patterns for human autoantibodies directed against the occupied hormone receptor. A and B represent those cases that had a decreased binding as a function of the concentration of hCG, while C and D show the opposite effect with an increase of binding. Curves B and D taper off with 10 U hCG/well, while A and C show a constant decrease or increase, respectively.

Figure 5. Gel permeation profiles of (A) the soluble (S80) and (B) the extractable (ST288) raw antigen preparations. The Superose 12 column (Pharmacia Fine Chemicals) was equilibrated with PBS, pH 7.2. Ultraviolet (UV) detection was done at 280 nm, 0.05 absorption units.

pregnant) we decided to use bovine ovaries. Within the ovary we chose to work with corpus luteum as an antigen since it corresponds to one of the IFL binding patterns. Furthermore, corpus luteum was the screening substrate for choosing sera to be used in the experiments that led to the demonstration of a

complement-mediated cytotoxic effect of serum on granulosa cells (29). From a theoretical and functional point of view corpus luteum extracts should provide a rather homogeneous material for the biochemical work.

We have been able to demonstrate that some patients with sterility can react in vitro with the LH/hCG receptor present in the corpus luteum. Binding of human autoantibodies to both the nonoccupied receptor and the receptor-hormone complex implies a functional relationship to antigenicity. Similar data were obtained by Heyma and Harrison (32) for the TSH receptor in a small-scale study involving only four patients with Graves' disease. Antibody binding could be occurring to both the receptor itself as well as to the exposed sites of the hormone. These sites could correspond to the β -chain as has been shown by Milius et al. (33). Methodological differences in trying to demonstrate antibody binding to the LH/hCG receptor between our approach and that of others (11, 14, 18) could explain the differing results. We feel that it is important to use a highly purified receptor preparation based on the combined use of lectins and LH-affinity chromatography. Besides the detection of anti-hormone receptor antibodies we also demonstrated that patients with infertility can present serum autoantibodies that are directed against other components of the corpus luteum.

Although some IgG subclasses were significantly associated with different antigen fractions, all IgG subclasses were detected. The predominance of IgG₁ and IgG₂ could be related to the biochemical nature of the antigens (34). Additionally, these antibodies presented both kappa and lambda light chains, thus revealing that the humoral immune response to ovarian antigens is a polyclonal one.

The results obtained for the group of patients with endometriosis is in accordance with previous data from Mathur et

Figure 6. Chromatofocusing of the extractable antigen fraction (ST288). The chromatographic procedure was done with the MONO P column according to the manufacturer for the pH range 6.3–4. The thin line represents the pH values of the corresponding UV peaks. The UV detection was done at 280 nm, AUFS 0.1. The solid bars beneath the chromatogram represent the number of positive cases that showed a positive binding to the corresponding fraction. Each millimeter of height represents one positive patient. Three assays (I, II, III) were plotted.

Table III. Main Protein Components and Terminal Sugar Residues of the S80 and ST288 Fractions after Gel Permeation Chromatography on Superose 12

Peak number	Area % of total	Apparent molecular weight	Terminal sugar residues*
80,000 g supernatant (S80)			
1	1.4	>500,000	1, 2, 3, 4, 7, 8
2	10.99	114,000	1, 2, 3, 4, 7, 8
3	16.91	67,000	1, 2, 3, 4, 7, 8
4	9.83	28,000	1, 2, 3, 4, 7, 8
5	10.99	5,500	4, 7, 8
6	21.50	1,100	4, 8
7	24.53	280	7, 8
288,000 g supernatant after Triton X-100 extraction of the 18,000 g pellet (ST288)			
1	26.84	>500,000	1, 2, 3, 4, 7, 8
2	12.71	36,000	1, 2, 3, 4, 7, 8
3	9.35	16,000	2, 3, 4, 7, 8
4	2.39	3,800	3, 4, 7, 8
5	5.56	1,100	3, 4, 7, 8
6	11.00	370	7, 8

* Terminal sugar residues determined with a lectin-peroxidase ELISA. 1, alpha-D-mannopyranoside and alpha-D-glucopyranoside; 2, methyl-2-acetamido-2-deoxy-D-galactose; 3, alpha and beta-D-linked 2-acetamido-2-deoxy-D-glucose; 4, D-galactopyranoside; 5, Gal-beta-(1 → 3)-GalNAc; 6, alpha- or beta-D-GalNAc; 7, alpha-L-fucose; 8, NAc-glucosamine.

al. (12), who also demonstrated the binding of antibodies to both the ovary and the endometrium, thus indicating that genital tract antigens are seen as targets in this disease. In contrast to these findings it is interesting to note that the use of IFL even at much lower serum dilutions did not allow identification of Ov-Ab in any of the patients with sterility (2). Those patients with autoimmune adrenal failure who were positive for adrenal antibodies on IFL were not always positive for Ov-Ab in the ELISA system. These results confirm again the observations of others of a lack of correlation between adrenal and Ov-Ab (6, 8, 11). The reason for this discrepancy could be either the time course of the disease, since not all antibodies are detectable at a single stage (6), or the presence of only anti-LH/hCG receptor antibodies (ovary specific). Although the number of patients with polyendocrinopathy was low, almost all of them were positive for Ov-Ab. Thus, when the clinical setting becomes more complex with multi-organ involvement, Ov-Ab can be found. Our finding of Ov-Ab in patients without evidence of a further autoimmune disease is in accordance with the results of others (10, 35).

We cannot provide a definitive interpretation on the possible role of Ov-Ab in the pathogenesis of sterility. It can only be speculated that they could interfere with ovarian function since immunosuppressive treatment of patients with Ov-Ab and infertility has led to a normalization of menses (7, 14–17). Experimental data are available on a complement-dependent cytotoxic effect on granulosa cells with a reduction of progesterone production when the serum from patients with Addison's disease, containing antibodies to corpus luteum, was added to granulosa cells in vitro (29).

In conclusion, antibodies directed against the corpora lutea of bovine ovaries can be positively detected in patients with infertility as well as in patients with polyendocrinopathy. Both the unoccupied receptor and the hormone-receptor complex act as antigens. The predominant IgG subclasses are IgG₁ and IgG₂ when tested in the assays that use soluble and extractable antigen fractions, respectively. Such antibodies appear to be specific for ovary since no inhibition of the binding reaction could be achieved using either thyroid or liver extracts. The antibodies recognize a glycoprotein antigenic complex in each antigen fraction. The involvement of the ovary as a target tissue has been demonstrated in cases of autoimmune oophoritis, infertility, and systemic lupus erythematosus (13, 17, 19, 22, 23). Thus, the scope of research in the field of immunology of reproduction has to be enlarged to include the corpus luteum and the LH/hCG receptor.

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