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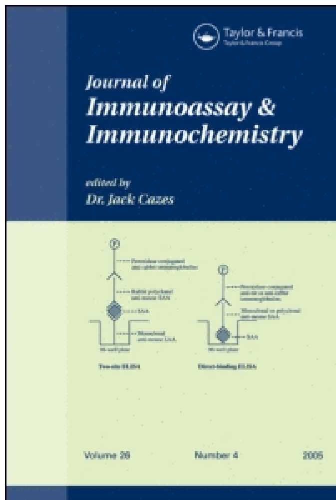

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Jung Gu Kim[a]; Byron E. Anderson[b]; Robert W. Rebar[a]; Andrew R. Labarbera[ac]
[a] Departments of Obstetrics and Gynecology, University of Cincinnati College of Medicine Cincinnati, Ohio [b] Departments of Physiology and Biophysics, University of Cincinnati College of Medicine Cincinnati, Ohio [c] Department of Cell, Molecular and Structural Biology Northwestern, University Medical School Chicago, Illinois

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JOURNAL OF IMMUNOASSAY, 12(4), 447-464 (1991)

A BIOTIN-STREPTAVIDIN ENZYME IMMUNOASSAY FOR DETECTION OF ANTIBODIES TO PORCINE GRANULOSA CELL ANTIGENS

Jung Gu Kim*, Byron E. Anderson+,
Robert W. Rebar* and Andrew R. LaBarbera*#
*Departments of Obstetrics and Gynecology
and #Physiology and Biophysics
University of Cincinnati College of Medicine
Cincinnati, Ohio 45267
and
+Department of Cell, Molecular and Structural Biology
Northwestern University Medical School
Chicago, Illinois 60611

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ABSTRACT

A colorimetric solid-phase enzyme immunoassay has been developed which quantifies antibodies to porcine granulosa cell membrane antigens in rabbits immunized with porcine granulosa cells. A cell-free, particulate membrane preparation of porcine granulosa cells was used as coating antigen. A biotinylated second antibody in conjunction with a streptavidin-β-galactosidase conjugate was utilized to amplify reactivity. The enzyme β-galactosidase was used due to high background obtained using peroxidase, presumably due to endogenous peroxidase activity of the tissue. Sigmoidal serum dilution curves were obtained with immune rabbit sera indicating that absorbance was related to the concentration of antibodies. Assay activity was reduced by preincubation of immune serum with granulosa cell membranes. Sera from ovariectomized or pre-immune rabbits did not yield any specific binding in the assay. This assay has potential applicability for quantifying antiovarian and anti-granulosa cell antibodies in women suspected of having autoimmune premature ovarian failure.

(Key Words: rabbit, pig, ovary, antibodies, galactosidase)

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INTRODUCTION

Hypergonadotropic hypoestrogenic amenorrhea in women is characterized by an inability of the ovary to respond to pituitary gonadotropins (1). Immunofluorescent and ligand-binding techniques have been used to demonstrate the presence of circulating antibodies to ovarian tissue in the sera of many women with this disorder, and the presence of antiovarian antibodies suggests that an autoimmune mechanism might be involved in the pathogenesis of this form of gonadal dysfunction (2). The immunocytochemical techniques have the disadvantages of being cumbersome and of yielding only qualitative results, and ligand-binding methods (3) require inconvenient and labile radioisotopic antibody preparations.

Since Van Weeman and Shuurs (4) first described enzyme-linked immunosorbent assay (ELISA), numerous modifications have been introduced to improve simplicity, safety and convenience of detection and quantification of antibodies. Incorporation of the biotin-avidin system, which originally was used in immunocytochemical staining of tissues, into ELISA procedures amplifies the colorimetric response, and thus increases the sensitivity of the assay (5). In some cases, ELISA is more sensitive than radioimmunoassay (5).

In the present study, we developed an ELISA which incorporates the biotin-streptavidin system to quantify antibodies to porcine ovarian granulosa cells in sera of rabbits immunized with porcine granulosa cell membrane antigens. This assay can be utilized not only in detecting antiovarian antibodies in animals with

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experimentally induced ovarian failure (6) but also has potential applicability in screening human patients with autoimmune ovarian failure for antiovarian antibodies (7).

MATERIALS AND METHODS

Granulosa Cells

Granulosa cells were harvested by needle aspiration (8) of immature small follicles (1-2 mm diameter) from ovaries of 3-6 month old pigs collected at a local slaughterhouse. Follicular fluid containing cells was centrifuged at 200 x g for 15 minutes and the pelleted cells were washed 3 times with ice-cold phosphate-buffered saline (PBS), pH 7.4.

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Particulate Membrane Preparations

Washed cells were resuspended in 10 vol of 10 mM Tris (Sigma Chemical Co., St. Louis, MO) buffer, pH 7.4 for 30 min and homogenized with six strokes of a motor-driven Teflon/glass homogenizer five times. Whole ovaries from pigs in the follicular phase of the estrous cycle were suspended in Tris buffer (100 mg tissue/ml), minced, and homogenized as for granulosa cells. A 20,000 x g membrane fraction was prepared from each homogenate as described previously (9) and the fractions stored at -70°C. Protein in cells and particulate membrane preparations was quantified using the BCA (bicinchoninic acid) protein assay kit (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA, Sigma Chemical Co.) as standard.

Detergent Solubilization of Membrane Constituents

Membrane constituents were solubilized at 4°C according to the method of Sperbeck and LaBarbera (10). Briefly, 20,000 x g porcine granulosa cell membranes were resuspended in the non-ionic detergent Triton X-100 (Pierce Chemical Co., Rockford, IL) in 10 mM Tris-buffered (pH 7.4) 0.14 M NaCl at a ratio of 25 mg membrane protein/ml of buffer. The suspension was sonicated 5 times with 15 sec pulses and extracted with 3 vol of ice-cold petroleum ether for 30 min. The buffer phase was removed, centrifuged at 100,000 x g for 1 h and the detergent concentration of the supernatant was reduced to 0.05% by extraction with SM-2 resin (0.33 g/ml; BioRad, Richmond, CA) for 30 min. The solubilized protein solution was stored at -70°C.

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Generation of Antisera

Mature New Zealand white rabbits were injected intradermally with either intact granulosa cells (10-15 mg protein) or 20,000 x g membranes (1.5-3.0 mg protein) suspended in 0.5 ml PBS and emulsified in 0.5 ml of Freund's complete adjuvant (Sigma). Booster injections with the initial dose of antigen were administered 4 weeks after initial immunization. Two weeks after booster injection, rabbits were bled via an ear vein and sera were collected and frozen at -20°C.

Denaturation of BSA

A 0.12 M solution of sodium periodate was prepared in 100 ml of 0.2 M dibasic sodium phosphate, 0.1 M citric acid buffer at 4°C

and was adjusted to pH 5.5 with NaOH. This solution was added with stirring to 100 ml of 16% (w/v) BSA. The solution was stirred for 2 h and dialyzed against three changes of ice-cold PBS.

ELISA Procedure

The wells of flat-bottomed microplates (Nunc, Denmark) were coated with either: 1) 20,000 x g porcine granulosa cell membrane preparation; 2) detergent-solubilized 20,000 x g porcine granulosa cell membrane proteins; or 3) 20,000 x g porcine ovarian membrane preparation in 0.1 M sodium carbonate buffer, pH 9.6. Alternate wells were coated with BSA to serve as negative controls. The plates were incubated overnight at 4°C, washed 3 times in PBS and coated with PBS containing 1% BSA and 0.02% sodium azide at 37°C for 60 min. One hundred μl of either test rabbit serum diluted with PBS (pH 7.3) containing 1% BSA, 0.02% sodium azide, 0.1% Tween-20 and 0.3% denatured BSA (dPBSAT), or dPBSAT only were added to each well. Plates were incubated for 2 h at 37°C and washed 4 times with PBS containing 1% BSA, 0.02% sodium azide and 0.1% Tween-20 (PBSAT). One hundred μl of a goat anti-rabbit immunoglobulin G (IgG)-biotin conjugate (Vector Laboratories, Burlingame, CA) diluted 1:2000 with dPBSAT were added to each well. Plates were incubated for 60 min at 37° and washed 4 times with PBSAT. One hundred μl of streptavidin-β galactosidase conjugate (Bethesda Research Laboratories, Rockville, MD) diluted 1:2000 with dPBSAT were added to each well. Plates were incubated for 60 min at 37°C and washed with PBSAT. One hundred μl of o-nitrophenylgalactose (ONPG, 4 mg/ml) dissolved in PBS and containing 0.77 mg/ml 2-β-mercaptoethanol were added as substrate to

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each well. Absorbance (405 nm) was determined using a Biotek microplate reader. Each plate included 8 wells which had received all reagents except serum and 8 wells which had only ONPG. Specific activity was calculated by subtracting the absorbance measured in wells coated with BSA ("nonspecific binding") instead of membrane antigen from the absorbance in wells coated with porcine antigen ("total binding"). Procedures were performed as above in duplicate unless otherwise specified.

In certain experiments the effect of poly-(L)-lysine (PLL) on the binding of 20,000 x g granulosa cell membranes was tested using immune rabbit serum. PLL-coated plates were prepared by adding 1 mg of PLL (molecular weight, 406,500; Sigma Chemical Co. #1524) in 100 μl of PBS. Plates were incubated for 30 min at room temperature and excess PLL solution was removed. Total binding in PLL-coated wells was similar to that in uncoated wells.

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RESULTS

Concentration of Coating Antigen

In order to determine the optimal concentration of coating antigen for maximal specific binding, plates were coated with 100 μl of 0.1-27 μg/ml of 20,000 x g granulosa cell membranes. At rabbit serum dilutions of both 1:100 and 1:1000, specific binding was related to the concentration of coating antigen up to an antigen concentration of 3 μg/ml, above which absorbance did not increase further. An antigen concentration of 3 μg/ml was used in subsequent experiments.

When the PLL-coated wells were used with 20,000 x g granulosa cell membranes and compared with non-PLL-coated wells, total binding was similar. Nonspecific binding, however, was significantly higher in coated wells than in uncoated wells ($p<0.001$). Therefore, wells were not coated with PLL in subsequent experiments.

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Concentrations of Biotinylated Second Antibody and Enzyme Conjugate

Optimal dilutions of each of the developing reagents were determined using plates coated with 20,000 x g granulosa cell membranes and a 1:1000 dilution of serum of a rabbit immunized with 20,000 x g granulosa cell membranes. Goat anti-rabbit IgG-biotin conjugate was tested at dilutions ranging from 1/500 to 1/4000; streptavidin β-galactosidase was tested at dilutions ranging from 1/500 to 1/8000. Maximal activity was attained using a 1:2000 dilution of goat anti-rabbit IgG-biotin conjugate with a 1:2000 dilution of streptavidin β-galactosidase (Table 1).

Incubation Time

Absorbance in the ELISA was related to the duration of incubation with immune rabbit serum, goat anti-rabbit IgG-biotin conjugate and streptavidin β-galactosidase conjugate. Plates were coated with 20,000 x g granulosa cell membranes and incubated with sera of rabbits immunized either with 20,000 x g granulosa cell membranes or with intact granulosa cells for 15-120 minutes. With both immune sera, specific binding occurred rapidly in the first 15 min of incubation, declined slightly and then remained constant for at least 2 h. A 15 min incubation with immune sera was used to

TABLE 1

Relative Activities at Different Dilutions of Biotinylated Goat Anti-Rabbit IgG and Streptavidin β-Galactosidase

Biotinylated Goat Anti-Rabbit IgG Dilution	Streptavidin β-Galactosidase Dilution				
	1:500	1:1000	1:2000	1:4000	1:8000
1:500	4.78	3.57	3.09	6.34	2.51
1:1000	4.80	4.15	4.03	3.74	2.38
1:2000	3.15	3.75	8.15	3.85	3.43
1:4000	3.74	4.30	4.50	4.22	2.81

* Note: Activities expressed as ratio of mean absorbance of duplicates of immune (8 wk) serum to mean absorbance of duplicates of preimmune serum

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study the time course of binding of biotinylated second antibody. After binding of the primary rabbit antibody, plates were incubated with goat anti-rabbit IgG-biotin conjugate for 15-120 min. Specific binding increased for 15 min after which it slowed and then remained constant after 30 min. Maximal specific binding was observed when plates with antigen-primary antibody-second antibody complex were incubated with the streptavidin β-galactosidase conjugate for 60 min after which binding did not increase any further. In subsequent experiments, incubation times for immune rabbit sera, goat antirabbit IgG-biotin conjugate and streptavidin β-galactosidase conjugate were 15 min, 30 min and 60 min, respectively.

Effect of Denatured BSA on Sensitivity of ELISA

In attempting to minimize nonspecific binding of immunological reagents in the ELISA, we determined that inclusion of denatured BSA

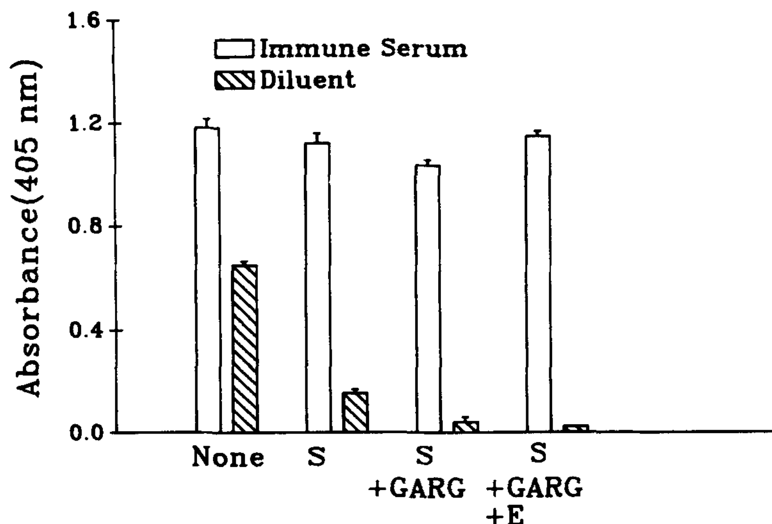

Figure 1. Effect of 0.3% denatured bovine serum albumin on sensitivity of ELISA. Either serum (1:1000 dilution) of a rabbit immunized with 20,000 x g porcine granulosa cell membranes or diluent buffer was incubated in wells coated with 20,000 x g porcine granulosa cell membranes. Denatured BSA (0.3%) was added in either the rabbit serum diluent ("S"); the diluents for the rabbit serum and goat anti-rabbit IgG-biotin conjugate ("S+GARG"); or diluents of rabbit serum, goat antirabbit IgG-biotin conjugate and streptavidin β-galactosidase ("S+GARG+E"). Bars represent means of quadruplicate determinations and vertical lines the standard deviations.

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in the reagent diluent at a concentration of 0.3% (w/v) substantially reduced nonspecific absorbance (Fig. 1). Plates were coated with 20,000 x g porcine ovarian membranes and incubated with either immune rabbit serum diluted 1:1000 with diluent or diluent alone. Inclusion of denatured BSA in the diluent for each reagent did not affect the absorbance in wells with immune rabbit sera but did reduce nonspecific absorbance in wells with diluent ($p<0.001$).

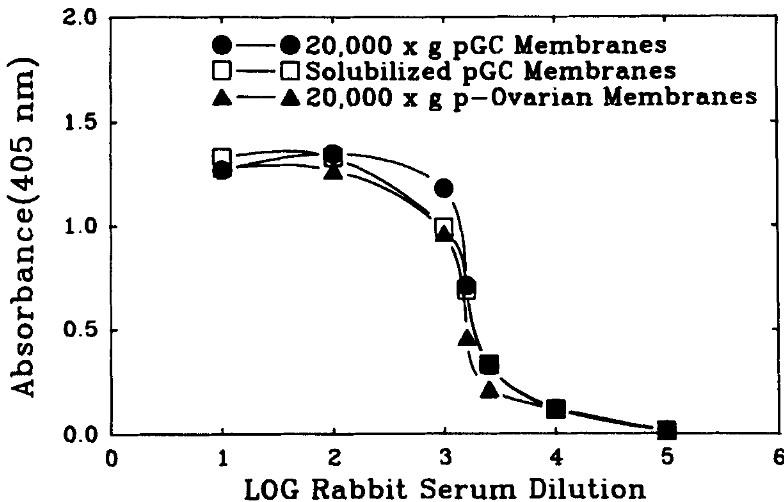

Figure 2. Comparison of different coating antigen preparations in ELISA. Immune serum (1:1000 dilution) was obtained from rabbit immunized with 20,000 x g porcine granulosa cell membranes. Wells were coated with either of three different antigens: 20,000 x g porcine granulosa cell membranes; a detergent-solubilized preparation of 20,000 x g porcine granulosa cell membranes; or a 20,000 x g particulate preparation of porcine ovarian membranes. Points represent means of duplicate determinations.

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Therefore, denatured BSA was included in the diluent for the primary immune sera, biotinylated secondary antiserum and enzyme conjugate.

Comparison of Different Coating Antigens

Wells were coated with either 20,000 x g porcine granulosa cell membranes, solubilized preparations of 20,000 x g porcine granulosa cell membranes, or 20,000 x g porcine ovarian membranes. There was a dose dependent relationship between the concentrations of rabbit serum IgG and binding to porcine antigens. ELISA with each of 3 porcine coating antigen preparations yielded similar

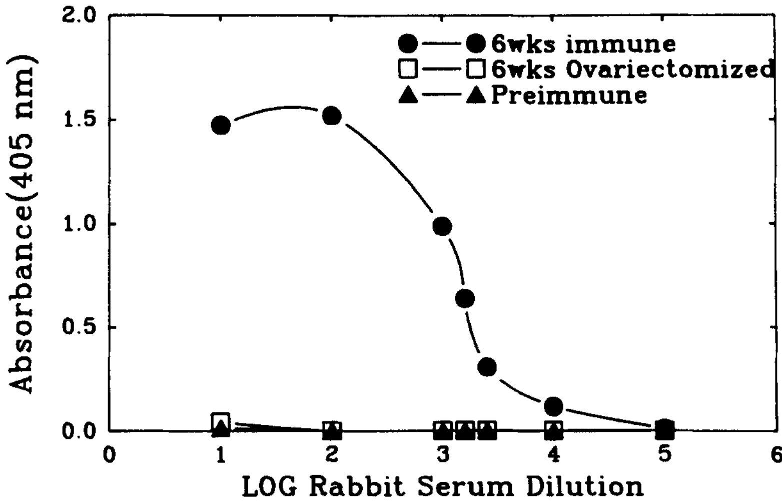

Figure 3. Dilution curves of preimmune, immune and ovariectomized rabbit sera in the ELISA. Rabbits were bled either prior to immunization ("preimmune"); 6 weeks after initial immunization with porcine granulosa cells ("6 wks immune"); or 6 weeks after ovariectomy ("6 wks ovariectomized"). Coating antigen was 20,000 x g porcine granulosa cell membranes. Values represent means of duplicate determinations.

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estimates of antiovarian antibody reactivity in immune rabbit sera when specific antiovarian antibody reactivity was defined as the highest dilution of immune serum which produced an absorbance greater than the mean absorbance + 3SD produced by sera from 18 different normal nonimmune rabbits (Fig. 2). Fig. 3 illustrates the dilution curve of serum of a rabbit 6 weeks after initial immunization with 20,000 x g porcine granulosa cell membrane preparations. Using the midpoint of the titration curve as a quantitative indication of reactivity the antiserum titer was at approximately a 1:1000 dilution. The absence of antibody binding

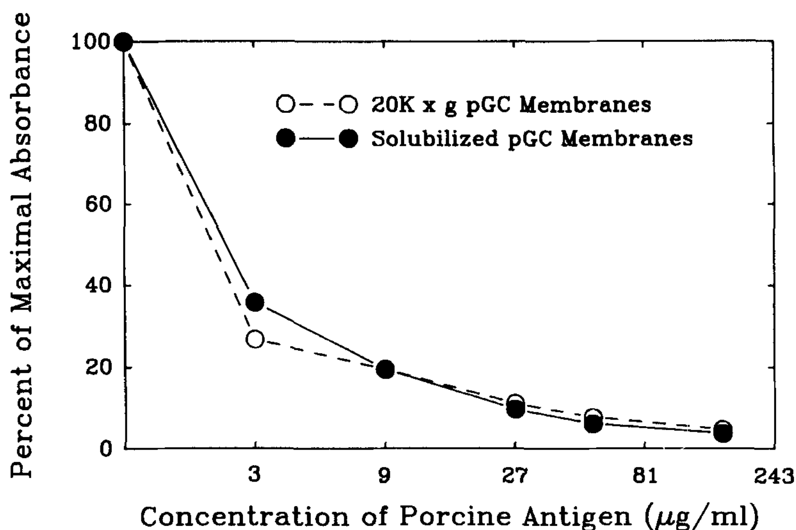

Figure 4. Binding inhibition test in serum (6 weeks after primary immunization) of a rabbit immunized with 20,000 x g porcine granulosa cell membranes. Serum (1:1000 dilution) was preincubated with increasing concentrations of either 20,000 x g porcine granulosa cell membranes or solubilized porcine granulosa cell membrane protein at 37°C for 15 minutes before application to ELISA plates. Points represent means of duplicate determinations.

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from 6 weeks ovariectomized rabbit sera and preimmune sera is also shown for 20,000 x g porcine granulosa cell membranes.

Specificity of ELISA

Binding specificity was confirmed by preincubating serum from a rabbit immunized with 20,000 x g granulosa cell membranes with either 20,000 x g granulosa cell membranes or detergent-solubilized granulosa cell membrane proteins. The preabsorbed sera were tested for binding by ELISA to either granulosa cell membranes or detergent-solubilized granulosa cell membrane proteins,

respectively, as coating antigen. Sera preincubated with increasing quantities of antigen yielded correspondingly lower binding in the ELISA (Fig. 4). Serum preincubated with 154 μg/ml membrane protein had <4% specific binding in subsequent ELISA with either of the two coating antigens.

DISCUSSION

This report describes optimal conditions for a solid phase enzyme immunoassay for estimation of titers of anti-porcine granulosa cell antibodies in sera of rabbits immunized with porcine granulosa cell protein. The assay utilizes a biotin-streptavidin system to amplify the absorbance due to the antibody-antigen reaction.

Streptavidin extracted from the bacterium *Streptomyces avidinii* has 4 high affinity sites for biotin. This biotin-streptavidin system leads to the amplification of the antigen-antibody reaction which in turn results in improved sensitivity. Streptavidin enzyme conjugates were found to be far superior to the egg avidin conjugates (11). As use of streptavidin-horseradish peroxidase conjugate resulted in high nonspecific background absorbance, presumably due to endogenous peroxidase activity in enriched antigen preparations (12,13), we used streptavidin β-galactosidase with no concern for endogenous enzyme activity.

The sensitivity of the assay system is suggested in the results of the antigen coating titration curve (Fig. 2) and the inhibition of antibody reactivity with addition of solution phase antigen preparations (Fig. 4). Near maximal activity of a 1:100

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dilution of the antiserum was obtained with an antigen coating of 1 μg/ml, 100 ng of antigen preparation per well (100 μl volume). Only a portion of the preparation may be assumed to be antigenic and so some fraction of the 100 ng antigen protein is being detected in the assay system. Also, 3 μg/ml (300 ng/100 μl) of antigen preparation in the solution phase yields nearly 80% inhibition of binding of antibody to solid phase coated antigen (Fig. 4) again indicating that small amounts of anitgen were being detected by the assay system.

Some investigators (13,14) used poly-(L)-lysine to increase absorption of coating antigen on wells. However, in our ELISA system poly-(L)-lysine seemed not to affect total binding activity but to increase nonspecific binding activity in control wells. dPBSAT as diluent of rabbit antisera, goat antirabbit IgG-biotin conjugate and streptavidin β-galactosidase decreased ELISA activity in wells with diluent without influence of specific binding activity in wells with immune rabbit sera, resulting in increase in sensitivity of the ELISA (Fig. 1). The denatured BSA used in the diluent may be blocking antibody reactivities in the rabbit antiserum which react with similar BSA epitopes exposed when the protein is adsorbed to the polystyrene microtiter plate wells (15).

We examined different incubation lengths for rabbit antisera to determine the optimal incubation time to induce maximal activity in the ELISA. A 15 minute incubation was optimal because after longer incubation times resulted in progressively greater nonspecific binding. In the present study, we found a 60 minute

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incubation for the biotin/streptavidin interaction to be optimal. Previous investigators reported that a 5 minute incubation of biotinylated reagents with avidin is not only possible but advantageous (6,16).

In our ELISA system, the degree of binding to coating antigen was related to the amounts of antiovarian antibody (Fig. 3). When serum from rabbits immunized with porcine membranes was preincubated with the same preparation as a coating antigen, its reactivity in the ELISA was almost completely abolished, indicating the specificity of the reaction (Fig. 4). Interestingly, ELISA with each of the 3 porcine coating antigen preparations yielded similar estimates of antiovarian antibody titer (Fig. 2). This may indicate that most antiovarian antibodies were induced against membrane proteins which could be solubilized with detergent. This can be further evidenced by the fact that in the binding inhibition test the degree of inhibition was similar for both serum preincubated with particulate granulosa cell membranes and serum preincubated with solubilized granulosa cell proteins.

The newly developed ELISA described in this report has advantages of simplicity, convenience and rapidity for estimation of antiovarian antibody titers. Importantly, the assay system is characterized by a high sensitivity and very low background (noise) values due to the construct of the assay and primarily to the addition of the denatured BSA to the antisera and other reagents used in the assay. Thus, this assay has the potential to be utilized in screening patients with apparent autoimmune ovarian

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failure for the presence of anti-granulosa cell antibodies. This assay has been utilized successfully to detecting antibodies to porcine granulosa cells in rabbits immunized with porcine granulosa cells (6). In those studies, changes in antibody titers correlated with changes in serum follicle-stimulating hormone concentration, an indirect indicator of ovarian function. In addition, women with premature ovarian failure were found to have higher titers of antiovarian antibodies than normally menstruating women using the ELISA described here (7).

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Address correspondence to Andrew R. LaBarbera, Ph.D., Department of Obstetrics and Gynecology, University of Cincinnati College of Medicine, 231 Bethesda Avenue (ML 526), Cincinnati, OH 45267-0526 USA.

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