Heteroimmunization with isolated pig zonae pellucidae

A. G. Sacco and V. S. Palm

Wayne State University School of Medicine, Department of Gynecology and Obstetrics, C. S. Mott Center, 275 E. Hancock Avenue, Detroit, Michigan 48201, U.S.A.

Treatment of zonae pellucidae with antiserum containing antibody activity against zona-specific antigens produces a precipitation layer on the outer surface of the zona (Ownby & Shives, 1972; Sacco & Shivers, 1973; Sacco, 1977) which inhibits both sperm attachment and penetration (see Shivers, 1974, for review). The presence of this precipitation layer is believed to be responsible for the observed inhibition of fertilization in vitro and in vivo following either antiserum treatment of eggs or passive immunization (Jilek & Pavlok, 1975; Oikawa & Yanagimachi, 1975; Tsunoda & Chang, 1976a, b, c). To date, all antiseras demonstrating anti-zona activity have been prepared by immunizing animals with ovary, unfertilized eggs or preimplantation-stage embryos (see Shivers, 1974, 1975 for review). These antiseras initially contain antibodies directed against many tissue components and must be rendered specific to the zona by absorption procedures. Such absorbed antiseras are obviously contaminated by components of the absorbing tissues which may interfere with or influence results from subsequent antifertility testing. We report here initial attempts to produce monospecific anti-zona pellucida serum by using isolated pig zonae obtained from unfertilized follicular eggs as the immunizing antigen. By the use of isolated zona material as the immunogen rather than ovary or intact eggs or embryos, the resulting antiserum may not require absorption to be monospecific for zona pellucida.

Methods

Collection of isolated pig zonae. Fresh pig ovaries were obtained from a commercial slaughterhouse. Follicular fluid was obtained by aspiration with a 1-ml tuberculin syringe and 26-gauge needle and the follicular eggs were collected from the pooled fluid under a stereomicroscope. Eggs were washed in several changes of 0.85% (w/v) NaCl and agitated in 0.5 ml of 0.01% (w/v) sodium citrate in 0.85% NaCl to remove cumulus cells. To obtain isolated zonae, the cumulus-free eggs were drawn into and expelled from a micropipette with an internal bore slightly less than the diameter of the egg plus its zona. The egg is destroyed during this procedure. Isolated zonae were collected and washed in 3 changes of 0.85% NaCl. The various stages of this procedure are shown in Plate I.

Immunization format and antiserum evaluation. A single, male rabbit was injected with approximately 2000 isolated pig zonae following the immunization procedure described by Vaitukaitis, Robbins, Nieschag & Ross (1971). The gamma-globulin fraction of the antiserum was prepared by (NH₄)₂SO₄ precipitation (Heide & Schwick, 1973) and, after dialysis against phosphate-buffered saline (PBS; 0.1 M-PO₄, pH 7.2), was used for all experiments.

Antibody activity against zona pellucida was established by formation of the antibody-produced precipitation layer on the outer surface of the zona after treatment of zona-encased pig eggs. Cumulus-free eggs were incubated for 10 min at room temperature in 0.1 ml of the gamma-globulin fraction of the antiserum or preimmune serum, washed in 0.85% NaCl, transferred to well slides and viewed by light microscopy.

Tissue specificity of the antiserum was tested by immunodiffusion procedures. Pig tissues for use in immunodiffusion analyses were obtained fresh from a commercial slaughterhouse and, after homogenization in PBS (50% w/v) and centrifugation (12,000 g), the supernatants were used as the antigenic preparations.

Antiserum to isolated pig zonae was absorbed with pig follicular fluid. The follicular fluid from fresh ovaries was centrifuged (12,000 g; 20 min), freeze dried and added to 'whole' antiserum (150 mg follicular fluid/ml antiserum). The mixture was left at 4°C overnight, centrifuged (12,000 g) and the gamma-globulin fraction of the supernatant was prepared by (NH₄)₂SO₄ precipitation. After
dialysis against 0·85% NaCl this preparation was used as follicular-fluid absorbed antiserum to pig zona pellucida. The effectiveness of the absorption was always checked by immunodiffusion of the absorbed antiserum against pig follicular fluid. The failure of precipitin bands to develop against the follicular fluid was interpreted as successful absorption.

Polyacrylamide gel electrophoresis in SDS. Polyacrylamide gels (0·15 X 5·5 cm; 5·6%; 0·375 m-tris–HCl, pH 8·9) containing 0·1% SDS were used for the electrophoretic separation of solubilized isolated zonae, unfertilized egg material and pig follicular fluid (Laemmli, 1970). Electrophoresis was conducted in 0·025 m-tris, 0·192 m-glycine buffer, pH 8·3, containing 0·1% SDS at 0·8 mA/gel at 100 V for 45 min.

To prepare the samples, isolated zonae were solubilized in the presence of 0·75 m-2-mercapto-ethanol in 0·2 m-ammonium bicarbonate buffer, pH 8·0 (total vol. 200 µl), at 37°C for 60 min and freeze dried in 100 µl volumes. The freeze-dried material was reconstituted in 5 µl of sample buffer (0·0625 m-tris-HCl, pH 6·8, 10% (v/v) glycerol in the presence of 0·1% SDS and 10 mm-DTT) and incubated at 37°C for 60 min. The sample volume applied to each gel was 2 µl and represented approximately 300 solubilized zonae.

The unfertilized egg material remaining in the bicarbonate buffer after removal of the isolated zonae, referred to as ‘egg protein’, was freeze dried and treated as were the zona preparations. Samples of egg protein applied to gels (2 µl) represented material from approximately 300 eggs.

Freshly obtained follicular fluid was diluted 1:40 with sample buffer, incubated at 37°C for 60 min, and 2 µl volumes applied to the gels.

Molecular weight markers were phosphorylase A, bovine serum albumin and pepsin (Worthington Biochemical Corp.). Gels were fixed and stained for protein (Coomassie blue; Weber, Pringle & Osborn, 1972) and carbohydrate (Fairbanks, Steck & Wallach, 1971).

Results and discussion

Treatment of pig eggs with antiserum obtained from the first and all subsequent bleedings resulted in the formation of a dense precipitation layer on the outer surface of the zona, indicating good antibody titre against zona pellucida (Pl. 2, Fig. 7). However, immunodiffusion analyses to test antiserum tissue specificity demonstrated that the anti-zona pellucida serum was not monospecific for zona since 1 or 2 precipitin bands were produced against 20 of the 21 pig tissues examined.

Follicular fluid-absorbed antiserum failed to react with all tissues tested except ovary for which a single precipitin band was produced (Pl. 2, Fig. 8), presumably because the ovary contained the zona-specific antigen. This antibody activity against zona pellucida was verified by the formation of the precipitation layer on the zona after treatment of pig eggs (Pl. 2, Fig. 9).

Analyses of isolated zona material by SDS–PAGE revealed at least 5 Coomassie blue staining bands which were arbitrarily numbered 1–5 based on their relative mobility from the origin (Pl. 2, Fig. 10). Bands 1, 4 and 5 were relatively sharp whereas Bands 2 and 3 were dispersed. Companion gels of follicular fluid and egg protein run simultaneously with the zona material demonstrated a minimum of 12 and 4 stained protein bands respectively (Pl. 2, Fig. 12). A band with a mobility identical to that of Band 1 was present in the follicular fluid and egg protein gels and bands with mobilities similar to those of Bands 3, 4 and 5 were present in the egg protein gel and possibly in the follicular fluid gel. Thus, 4 of the 5 protein staining bands identified in the isolated zona pellucida gel had counterparts of similar mobility in either the follicular fluid and/or egg protein gels, suggesting, and supported by the immunological data, that the reduced components represented by these bands are probably follicular fluid and/or egg protein contaminants present in the isolated zona preparation. In view of the extremely fibrous and porous nature of the zona pellucida, as revealed by SEM (Dudkiewicz, Shivers & Williams, 1976), the washing procedures used were almost certainly insufficient for removal of such contaminants. More thorough washing procedures are probably unlikely to be more successful and would only result in the loss of greater numbers of isolated zonae.

The region represented by Band 2 in the isolated zona pellucida gel appeared to have no direct counterpart in the other gels, although several bands in the follicular fluid gel did migrate to the immediate area of Band 2, and Band 2 may therefore represent a protein present in zona pellucida.
The dispersed region of this band was estimated by extrapolation to be in a molecular weight range of 120,000–150,000 and contained PAS-positive material (Pl. 2, Fig. 11). The association of PAS stained material with this band is in agreement with reports that the zona consists primarily of mucopolysaccharides or glycoproteins (Braden, 1952; Jacoby, 1962; Löwenstein & Cohen, 1964) and the diffuseness of this staining is typical of the microheterogeneity characteristic of many glycoproteins (Gottschalk, 1966). We conclude that the best approach for producing a monospecific anti-zona pellucida serum by a method which does not require absorption would be by immunization with a biochemically purified zona preparation, and studies are in progress to elucidate the biochemical nature of the zona pellucida and zona antigen.

This work was supported in part by NIH General Research Grant No. RR-05384 to A.G.S. and by a fellowship of the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, grant No. Pa 200/3-4 to V.S.P.

References


Received 5 April 1977
EXPLANATION OF PLATES

Plate 1

Various stages in the procedure used to obtain isolated pig zonae pellucidae from follicular eggs.

Fig. 1. Fresh pig ovaries demonstrating follicles and 1 ml tuberculin syringe with 26 g needle used to aspirate follicles.

Fig. 2. Follicular eggs (arrows) as observed in follicular fluid. ×68.

Fig. 3. Pooled eggs surrounded by cumulus cell masses after collection from follicular fluid. ×68.

Fig. 4. Eggs after cumulus cell removal in 0.01% sodium citrate. ×68.

Fig. 5. Isolated zonae pellucidae before washing. ×68.

Fig. 6. Isolated zonae pellucidae after washing in 0.85% NaCl. ×68.

Plate 2

Fig. 7. Unfertilized pig eggs with exposed zonae treated with (bottom) antiserum (from rabbit, first bleeding) to isolated pig zonae pellucidae and (top) rabbit preimmune serum. Note the dense precipitation layer (arrow) on the zona of the antiserum-treated egg. ×960.

Fig. 8. Immunodiffusion plate showing reaction of follicular fluid-absorbed antiserum to isolated pig zonae pellucidae with pig ovary. A single precipitin band is produced between the antiserum and pig ovary while no bands are produced against the other reagents. Centre well, absorbed antiserum; Wells 1 and 2, pig ovary; Well 3, pig serum; Well 4, pig plasma; Wells 5 and 6, pig follicular fluid.

Fig. 9. Pig eggs with exposed zonae treated with follicular fluid-absorbed antiserum to isolated pig zonae pellucidae (upper left) and preimmune serum (lower right). Note the dense precipitation layer (arrow) on the zona of the antiserum-treated egg. ×960.

Fig. 10. SDS–PAGE gel of isolated pig zonae pellucidae stained for protein with Coomassie blue. Five bands numbered 1–5 starting from the origin are present.

Fig. 11. PAS-stained gel of isolated pig zonae pellucidae. Arrow indicates PAS-positive material in the region of Band 2.

Fig. 12. SDS–PAGE comparing (A) isolated pig zonae pellucidae preparation, (B) pig follicular fluid, and (C) egg protein preparation. All gels stained for protein with Coomassie blue. Arrows indicate the protein bands.