

Monoclonal Antibodies as Probes of the Distribution of ZP-2, the Major Sulfated Glycoprotein of the Murine Zona Pellucida

IAIN J. EAST and JURRIEN DEAN

Laboratory of Chemical Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT Three sulfated glycoproteins (ZP-1, ZP-2, and ZP-3) make up the zona pellucida, an extracellular glycocalyx that surrounds mouse oocytes. We have produced five monoclonal antibodies specific to the zona. All five immunoprecipitated ZP-2, and in addition, two of the antibodies immunoprecipitated ZP-3. This suggests the presence of either a common antigenic site or one made up in part by each of the two glycoproteins. The monoclonal antibodies bound to $\sim 1.3 \times 10^8$ binding sites per ovulated mouse egg which represents 2% of the total number of ZP-2 molecules present in the zona. ZP-2 appeared to be present throughout the zona and indirect immunofluorescence revealed a fibrous pattern with no evidence of localization. Furthermore, this pattern of distribution, which was identical for all five monoclonal antibodies, remained constant after fertilization at the two-cell embryo stage. Laser photobleaching demonstrated that ZP-2 is stably integrated in the extracellular matrix of the zona pellucida. No mouse tissue other than the ovary contained ZP-2 and ZP-2 is antigenically distinct from other previously described extracellular matrix proteins.

The zona pellucida is an acellular sheath that surrounds the mammalian oocyte and is thought to contain the species-specific sperm receptor. Following fertilization the zona is modified in the "hardening reaction" to prevent polyspermy and may also play a role in protecting the embryo as it passes down the oviduct (29). The murine zona is comprised of three sulfated glycoproteins designated ZP-1, ZP-2, and ZP-3 with average molecular weights of 185,000, 140,000, and 83,000, respectively (1, 3, 25). ZP-2 appears to be the major protein of the murine zona pellucida and is biochemically modified following fertilization (1, 4, 25). ZP-3 induces the sperm acrosome reaction and has been proposed as the species-specific sperm receptor (2). No function has been ascribed to ZP-1. The developmentally regulated synthesis of all three proteins occurs only during oogenesis and ceases prior to ovulation.

We have previously reported that the three sulfated glycoproteins of the zona pellucida are synthesized and secreted to form an extracellular matrix in which they are noncovalently linked to one another (25). However, little is known about

the distribution of the individual proteins within the zona pellucida, their tissue specificity, or whether or not they are related to other extracellular matrix glycoproteins (6, 28). During oogenesis cross sections of the zona appear by transmission electron micrographs to be homogeneous with the outer region having the appearance of several layers of loosely woven filamentous components (7, 26). Lectins have been used to probe the structure of the zona. Concanavalin A binding sites on the zona are evenly distributed although an asymmetric distribution has been noted with other lectins (8, 18). However, only recently have the lectin binding specificities of the individual zona glycoproteins been determined and only Concanavalin A and peanut agglutinin appear to distinguish among the zona proteins (24).

Monoclonal antibodies (14) can provide reagents with unique binding to individual proteins of the zona pellucida. We describe for the first time, the development of monoclonal antibodies specific to ZP-2, the major zona protein. These immunologically specific probes were used in studies designed to assess the tissue specificity of the major zona protein, its

relation with other known extracellular matrix proteins, as well as the distribution of ZP-2 within the zona pellucida itself.

MATERIALS AND METHODS

Isolation of Zona Pellucidae: DBA/2 mice were killed by CO₂ inhalation; their ovaries were removed and placed in ice-cold HEPES modified Brinster's medium (11) containing 0.2% NP-40, 10 µg/ml lima bean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml ethylenediamine tetracetic acid. Zona pellucidae were isolated as previously reported (8) with the following modifications: the ovaries were transferred to a wire screen (100 mesh), homogenized with a flattened glass rod, washed through a 210-µm Nylon screen, and a mixture of oocytes and zonae was collected on a 44-µm screen. The 44-µm screen was removed, rinsed in 5 ml of the above buffer, and the eluate freeze-thawed twice in an alcohol-dry ice bath prior to centrifugation (1,600 g, 30 min, 4°C) through 50% sucrose. The pellet was suspended in 3 ml of the Brinster's medium, and zonae were collected manually with a micropipette. Zonae were stored at -70°C.

Radioiodination: The frozen zonae were thawed, pelleted in a microfuge (12,000 g, 5 min), and the supernatant solution was discarded. The zonae were washed five times with PBS (1.5 ml) to remove all traces of lima bean trypsin inhibitor, resuspended in PBS, and solubilized by heating for 20 min at 60°C. The solution was clarified in a microfuge (Beckman Instruments, Inc., Palo Alto, CA) and the supernatant solution was retained.

The solubilized zonae were iodinated (5) and desalted on Sephadex PD-10 columns (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) that were equilibrated in PBS containing 0.2% gelatin. After dialysis against PBS, the iodinated zona proteins were stored in aliquots at -70°C. The monoclonal antibodies were iodinated and collected by the same procedure.

Immunization: Male rats (Osborne-Mendel) were injected intraperitoneally with a suspension of 1,000 zonae (5 µg of protein) in PBS emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, MI). Two further injections containing 5 and 20 µg, respectively, were given at 14-d intervals. After 6-8 wk, the rats received an intravenous injection of 10,000 zonae (50 µg) suspended in PBS (500 µl). Three days later, the rats were sacrificed by chloroform inhalation, the spleens removed for hybridization, and the blood collected by cardiac puncture and treated as described (13). The resultant antiserum was used as a positive control in all radioimmunoassays and immunofluorescence assays. Sheep (anti-rat Ig) antibodies were produced by injecting two sheep with the Ig fraction of preimmune rat serum (9).

Production, Screening, and Isotyping of Monoclonal Antibodies: Immunized rat splenocytes were fused to SP2/0 mouse myeloma cells (9) using polyethylene glycol 1000 (J. T. Baker Chemical Co., Phillipsburg, NJ). Cell lines secreting antibodies specific to the murine zona pellucida were identified by radioimmunoassay. Culture fluid (50 µl) was incubated for 4 h at 25°C with [¹²⁵I]zona pellucida (30,000 cpm, 100 µl) and 1% preimmune rat serum in PBS containing 1% NP-40 and 10 µg/ml dextran sulphate (50 µl). Sheep anti-rat Ig (100 µl) was then added and the tubes incubated at 4°C for an additional 16 h. PBS containing 1 M NaCl and 1% NP-40 (3 ml) was added, the tubes were vortexed, and then centrifuged (2,000 g, 4°C, 30 min). The supernatant solution was decanted and the tubes were drained on absorbent paper (15 min) prior to being counted in a Tracor gamma counter (model 1185, Tracor, Inc., Austin, TX).

Each cell line that secreted an antibody specific for the murine zona pellucida was cloned by limiting dilution (21) without the use of feeder cells. Positive clones (identified using the assay described above), were subjected to a second round of cloning. Antibody was produced in large amounts by generation of ascites tumors in NIH nude mice (21). Aliquots of ascites fluid were chromatographed on a Sephacryl S-200 column (1.5 × 110 cm) (Pharmacia Fine Chemicals, Inc.) equilibrated in 0.1 M Tris pH 8.0 containing 0.5 M NaCl. The IgG peak was pooled and rechromatographed on the same column. The monoclonal antibodies were stored frozen at -70°C. The heavy chain class of each monoclonal antibody was determined by replacing the second antibody in the radioimmunoassay described above with a range of class specific second antibodies (Miles Laboratories, Inc., Elkhart, IN) or with 200 µl of a 1:10 dilution of Pansorbin (Calbiochem-Behring Corp., La Jolla, CA).

Gel Electrophoresis: Gels were made and run as described by Laemmli (15). Purified antibodies were analyzed on two-dimensional gels using either isoelectric focusing or nonequilibrium, pH gradient electrophoresis in the first dimension (19, 20). The immunoprecipitation with monoclonal antibodies was performed as described (12), except that sheep anti-rat IgG (Miles Laboratories, Inc.) was used as a second antibody.

Binding to Superovulated Eggs: Groups of 40 ovulated eggs were incubated with fetal calf serum (50 µl), PBS (50 µl), and increasing amounts of

[¹²⁵I]monoclonal antibody (50 µl, 16 h, 4°C). The eggs were then washed four times with PBS containing 1% NP-40 (1.5 ml) and the bound radioactivity was determined. Competitive binding studies were done in the presence of 1,000-fold mass excess of unlabeled monoclonal antibody.

Immunofluorescence: Ovulated eggs or two-cell embryos were obtained from mice 16 and 40 h, respectively, after mating NIH Swiss mice (25). After incubation with antibody (100 µl, 30 min, 25°C), the eggs and/or embryos were washed four times in PBS, pH 7.4 (1.5 ml) and collected by micropipette. The eggs/embryos were then incubated with rhodamine-conjugated and affinity-purified-sheep anti-rat IgG (Jackson ImmunoResearch, Avondale, PA) (100 µl of a 1:10 dilution, 30 min, 25°C), washed, and suspended in 50 µl of PBS. The suspended eggs or two-cell embryos were transferred to a microscope slide. The cytoplasm was extruded from the zona by placement of a coverslip on the sample and the sample viewed using a Zeiss fluorescence photomicroscope III. Photographs were taken using Kodak Tri-X Pan film. Laser fluorescence photobleaching recovery (27) using fluorescein isothiocyanate labeled IE-3 (10) monoclonal antibody was kindly performed by Dr. Michael Eriden (The Johns Hopkins University, Baltimore, MD).

Frozen sections of mouse tissue were incubated with 50 µl of the anti-ZP-2 monoclonal antibodies diluted 1:10 in fetal calf serum. After washing by immersion in PBS, the tissue was stained with fluorescein-conjugated sheep anti-rat IgG diluted 1:20 in fetal calf serum. Frozen sections of skin (as a positive control) and ovary were stained with rabbit antisera specific for entactin (diluted 1:10, gift from Dr. Albert Chung, University of Pittsburgh, PA), laminin (1:5), fibronectin (1:20), collagen IV (1:10), or heparan sulfate (neat) (gifts from Drs. George Martin and Hynda Kleinman, National Institute of Dental Research, National Institutes) and visualized with a sheep anti-rabbit fluorescein isothiocyanate-conjugated second antibody. Immunoprecipitation of [¹²⁵I]-zonae with each antiserum, diluted as above, was performed according to a previously described method (12).

RESULTS

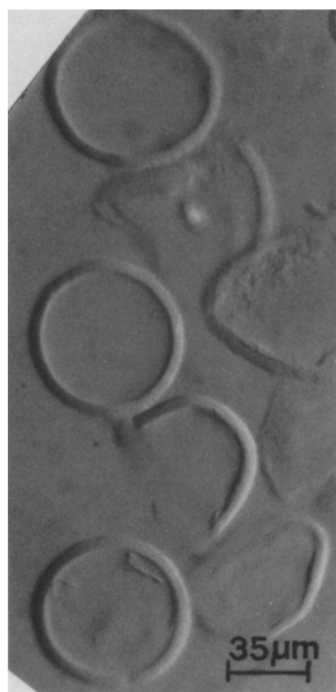
Production of Monoclonal Antibodies

Routinely 2,000-2,500 zonae pellucidae were isolated from 50 mice. By phase-contrast microscopy, the zonae appeared free of follicle cells and other cellular debris (Fig. 1A). Gel electrophoresis of iodinated zonae followed by autoradiography demonstrated three broad bands (Fig. 1B) representing ZP-1, ZP-2, and ZP-3 as previously observed (1, 25). Particulate zonae were used to immunize several rats. After three injections, the rats were bled and their sera tested for the ability to bind to [¹²⁵I]zonae. Those rats producing antizona antibodies were boosted intravenously with 50 µg of zonae and their spleen cells were used to make monoclonal antibodies.

In all, five cell fusion experiments were completed. The first three resulted in the production of no cell lines that secreted antibody specific for the murine zona pellucida. The fourth fusion resulted in one such cell line (IE-1). In the fifth fusion, 297 of 300 wells seeded yielded a positive result in the radioimmunoassay used to screen the parental wells. Of these, 100 were selected, grown, and subsequently stored frozen. Five clones were chosen at random and cloned twice by limiting dilution and then grown as ascites tumors in pristane primed NIH nude mice.

The IgG fraction of each ascites fluid was isolated by chromatography on Sephacryl S-200. Each antibody was characterized as to its heavy chain subclass and its ability to bind to protein A (Table I). As a further criteria of homogeneity and uniqueness, each purified monoclonal antibody was analyzed by two-dimensional gel electrophoresis. In each case, the combined pattern of the heavy chain microheterogeneity and a single light chain was unlike any other (Fig. 2). In four cases, only a single light chain was observed, which is a further indication that each cell line is truly monoclonal. The light chain of IE-4 which does not enter the isoelectric focusing gel was visualized as a single protein spot using a nonequilibrium pH gradient in the first dimension (data not shown).

A



B

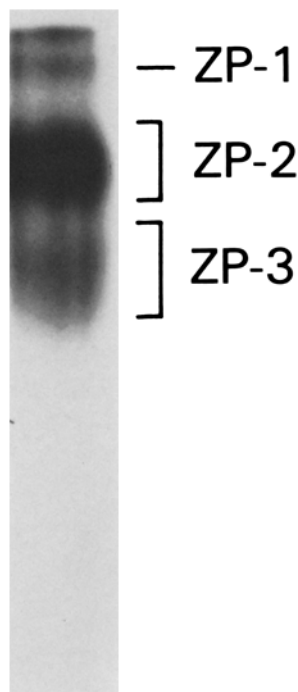


FIGURE 1 (A) Nomarski photomicroscopy of isolated zonae pellucidae. $\times 220$. (B) SDS gel electrophoresis of isolated zonae pellucidae after labeling with ^{125}I -Bolton and Hunter reagent.

TABLE I

Characteristics of Monoclonal Antibodies to the Murine Zona Pellucida

Hybridoma line	Isotype	Protein A binding	K_{diss} mol l^{-1}	Target antigen	Epitope
IE-1	IgG _{2a}	+/-	6×10^{-10}	ZP-2	A
IE-2	IgG _{2a}	-	3×10^{-10}	ZP-2	B
IE-3	IgG _{2a}	-	2×10^{-10}	ZP-2	B
IE-4	IgG ₁	+	3×10^{-9}	ZP-2/3	C
IE-6	IgG ₁	+	5×10^{-10}	ZP-2/3	C

Immunogenicity of Individual Proteins in the Zonae Pellucida

Aliquots of SDS-solubilized [^{125}I]zona proteins were incubated with a polyclonal antiserum and each of the monoclonal antibodies. The immunoprecipitates were analyzed by gel electrophoresis and autoradiography. The polyclonal antiserum immunoprecipitates zona proteins ZP-2 and ZP-3 but not ZP-1. No labeled material was detected in the immunoprecipitate formed with preimmune rat serum. Monoclonal antibodies IE-1, IE-2, and IE-3 bind specifically to ZP-2 (Fig. 3). Interestingly, IE-4 and IE-6 immunoprecipitate both ZP-2 and ZP-3. The criteria used to assure monoclonality effectively rule out the possibility that either the IE-4 or IE-6 cell lines is a mixture of two cell lines secreting different antibodies, one to ZP-2 and the other to ZP-3. Thus, IE-4 and IE-6 must bind to an antigenic determinant common to both glycoproteins, or to a site comprised of regions of both ZP-2 and ZP-3.

Little is known about the number or distribution of ZP-2 molecules in the zona pellucida. Therefore, each of the monoclonal antibodies was iodinated and utilized in a direct binding assay with ovulated eggs. The data were used to obtain a Scatchard plot (23) for each monoclonal antibody (Fig. 4). By the criteria discussed

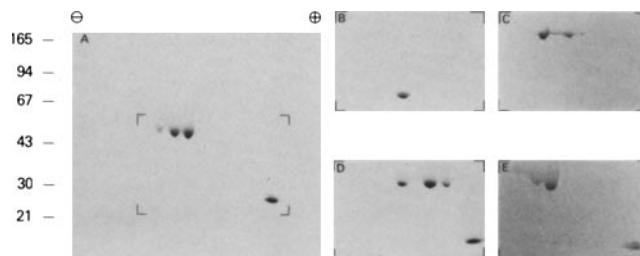


FIGURE 2 O'Farrell two-dimensional gels of purified monoclonal antibodies. Isoelectric focusing between pH 7.0 and 4.5 in the first dimension and SDS PAGE in the second dimension. (A) Monoclonal antibody IE-2 (brackets indicate region of gel shown in B-E). Values at left are the molecular weight $\times 10^3$. (B) IE-1; (C) IE-4; (D) IE-3; and (E) IE-6.

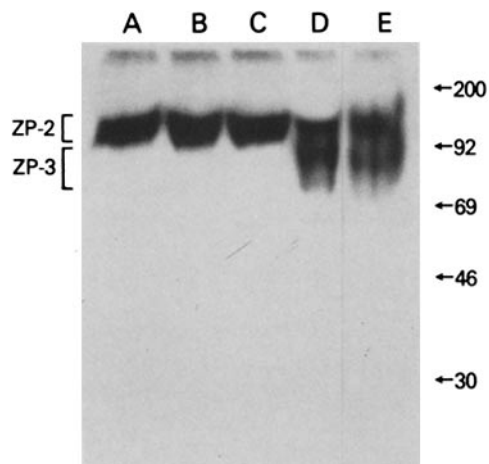


FIGURE 3 SDS gel electrophoresis of immunoprecipitates of solubilized ^{125}I -zona proteins by monoclonal antibodies. (A) IE-1; (B) IE-2; (C) IE-3; (D) IE-4; and (E) IE-6. Values at right are the molecular weight $\times 10^3$.

above each antibody appears to be monoclonal. Thus, straight lines were fitted to each Scatchard plot and had correlation coefficients between -0.93 and -1.00 . Dissociation constants calculated from the slopes of the lines are listed in Table I and ranged from 3×10^{-9} to 2×10^{-10} mol l^{-1} . Using the Scatchard analysis, the average number of ZP-2 molecules accessible to the antibodies was determined to be 1.3×10^8 per zona. This represents 2% of the total ZP-2 molecules per zona based on earlier estimates that the zona pellucida has a mass of 5 ng, that 47% of the zona consists of ZP-2, and that ZP-2 has an average molecular weight of 140,000 daltons (1, 25). This number of antibodies on a zona of 80- μ m diam represents one antibody on every 165 nm^2 . The binding of further antibody molecules may be prevented by steric hindrance of antibodies already bound.

To determine whether multiple antigenic determinants on ZP-2 were present on the exposed surfaces of the zona, an inhibition binding assay was designed to study the competition of antibodies for particular epitopes on the zona pellucida. Table II shows the ability of each unlabeled monoclonal antibody to inhibit the binding to ovulated eggs of each iodinated monoclonal antibody. Apart from inhibition of binding with the homologous antibody, only monoclonal antibodies IE-2/IE-3 and IE-4/IE-6 demonstrated mutual inhibition, which indicates that each pair binds to the same epitope. The grouping of the monoclonal antibodies based on their epitope

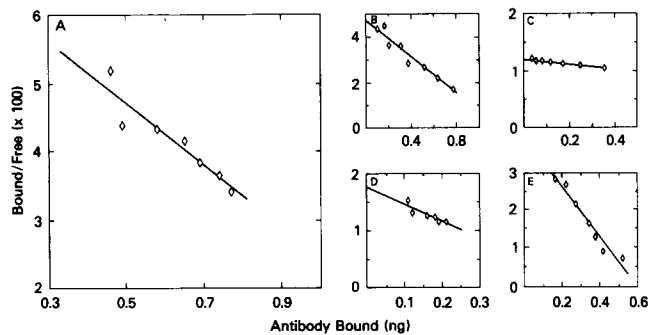


FIGURE 4 Binding of ^{125}I -monoclonal antibodies to ovulated eggs plotted according to Scatchard. (A) IE-3; (B) IE-1; (C) IE-4; (D) IE-2; and (E) IE-6.

specificity is summarized in Table I, where *A*, *B*, and *C* are used arbitrarily to designate the three epitopes. Thus, there are at least three antigenic determinants on ZP-2 accessible on the surface of the zona. Furthermore, the ability of each specific monoclonal antibody to bind both ovulated eggs and two cell embryos (see below), suggests that these three determinants are not modified by the known biochemical changes associated with fertilization (4).

Distribution of ZP-2 in the Zona Pellucida

The distribution of ZP-2 in the zona pellucida was determined using direct immunofluorescence of ovulated eggs. Monoclonal antibodies specific to ZP-2 (IE-1, IE-2, and IE-3) or monoclonals that bind to ZP-2 and ZP-3 (IE-4 and IE-6) completely stain the zona with no evidence of localization (Fig. 5*A*). The fibrous pattern of fluorescence, best seen at higher magnification in Fig. 5*B*, was similar with each monoclonal antibody tested. Staining of frozen ovarian sections with anti-ZP-2 monoclonal antibodies showed a uniform distribution of fluorescence throughout the width of the zona (Fig. 7*A*), which is further evidence of the even distribution of ZP-2 within the zona pellucida. Furthermore, ZP-2 appears to be stably integrated in the zona pellucida as determined by fluorescence photobleaching recovery. Fluorescein isothiocyanate-labeled anti-ZP-2 monoclonal antibodies were bleached to 40% of initial fluorescence and during a recovery phase

TABLE II
Binding of ^{125}I -Monoclonal antibodies to Ovulated Eggs in the Presence of Competing Monoclonal Antibodies

Labeled monoclonal antibody	Competing monoclonal antibody*					
	Nil	IE-1	IE-2	IE-3	IE-4	IE-6
IE-1	100	0	98	87	114	94
IE-2	100	116	0	3	171	133
IE-3	100	98	0	0	88	88
IE-4	100	109	135	76	0	6
IE-6	100	105	114	98	0	0

* Expressed as percent of control.

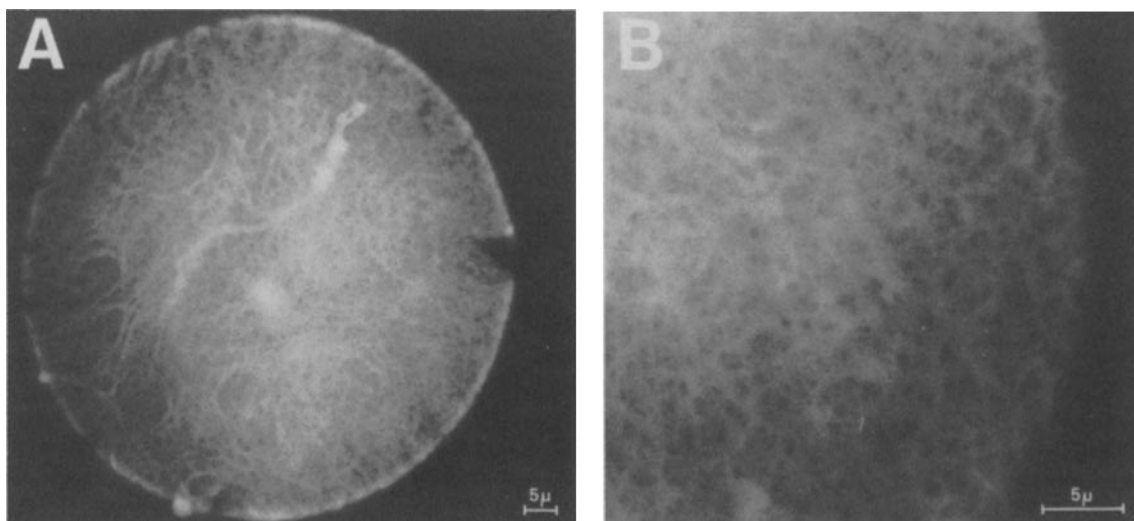


FIGURE 5 Indirect immunofluorescence microscopy of zona pellucida. (A) Ovulated eggs stained with monoclonal antibody IE-3 and fluorescein-conjugated sheep anti-rat IgG. (B) Ovulated eggs stained with monoclonal antibody IE-3.

there was no return of fluorescence to baseline (see Fig. 6). Thus, there is no evidence of lateral diffusion of ZP-2 in the zona pellucida, although it appears that there must be sufficient flexibility in the zona to accommodate the substantial increase of size of the enclosed oocyte during the preovulatory growth phase.

Similar immunofluorescence studies were conducted on the zonae of two-cell embryo. Although at the two-cell stage the functional and biochemical properties of the zona are known to be different from those of the ovulated eggs (2, 4), there was no change in the distribution or the fibrous pattern of ZP-2 in the zona as detected with our monoclonal antibodies (data not shown).

Tissue Localization and Specificity of ZP-2

The zona pellucida is an extracellular structure comprised of three sulfated glycoproteins. Other glycoproteins such as

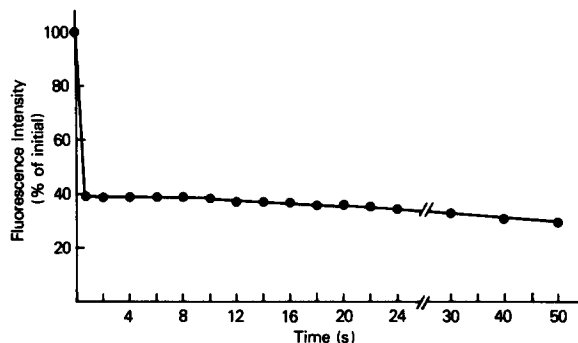


FIGURE 6 Fluorescence photobleaching recovery. Fluorescein isothiocyanate-monoclonal antibody IE-3 was incubated with ovulated eggs. Following initial laser bleaching, no recovery of fluorescence was observed.

laminin, fibronectin, and entactin have been demonstrated to participate with collagen IV in various extracellular matrices. Thus, we determined the distribution of ZP-2 in various mouse tissues. Using indirect immunofluorescence and monoclonal antibodies specific for ZP-2, we stained frozen sections of skin, brain, thyroid, heart, lung, pancreas, liver, gut, kidney, ovary, and uterus. No tissue other than the ovary stained positively for ZP-2. In the ovary, the indirect immunofluorescence was localized to the zona pellucida surrounding growing oocytes (Fig. 7A) and was not visualized in the basement membrane separating the thecal cell layers from the follicle.

Conversely, polyclonal antisera specific to collagen IV, laminin, or entactin did not stain the zona pellucida of mouse ovarian sections (Fig. 7, B-D), although all three of these extracellular matrix proteins are detected in the basement membrane surrounding the ovarian follicle. In a radioimmunoassay using heat solubilized zonae pellucidae, only anti-ZP-antibody immunoprecipitated [¹²⁵I]zonae. The labeled zonae were not precipitated by polyclonal antisera specific for laminin, fibronectin, entactin, collagen IV, or heparan sulfate. Thus, the major sulfated glycoprotein of the zona pellucida, ZP-2, is found only in the ovary and does not appear to participate in other extracellular matrices. Furthermore, ZP-2 appears to be a unique sulfated glycoprotein that is not antigenically related to previously described extracellular matrix glycoproteins.

DISCUSSION

We have mapped the distribution of the major sulfated glycoprotein of the murine zona pellucida using monoclonal antibodies specific to ZP-2. ZP-2 appears to be diffusely distributed on the surface of the zona and present throughout its thickness. At least three different determinants of ZP-2 are

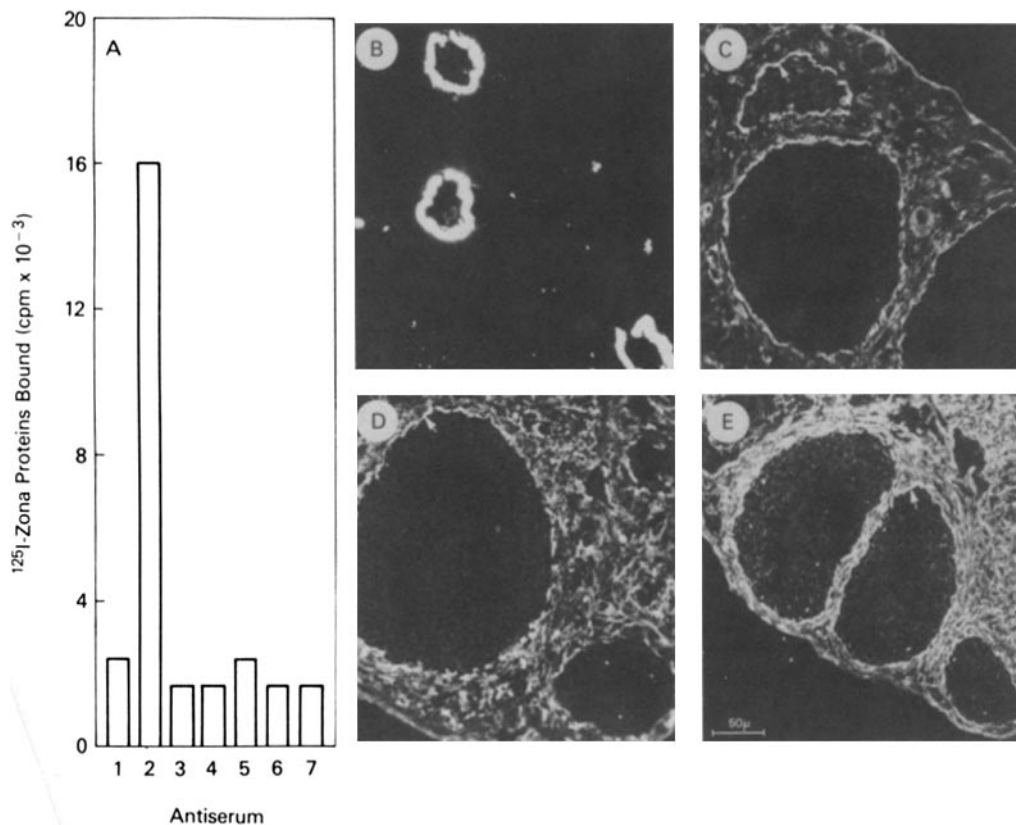


FIGURE 7 Immunoreactivity of zonae pellucidae. (A) Immunoprecipitation of ¹²⁵I-zonae pellucidae with (1) rat preimmune serum, (2) anti-ZP-2 monoclonal antibody, (3) anti-entactin antiserum, (4) anti-laminin antiserum, (5) anti-fibronectin antiserum, (6) anti-heparan sulfate antiserum, and (7) anti-collagen IV antiserum. Ovarian frozen sections stained with (B) anti-ZP-2 monoclonal antibody, (C) anti-entactin antiserum, (D) anti-laminin antiserum, and (E) anti-collagen IV antiserum. Arrows (C-E) indicate the follicular basement membrane.

exposed on the surface of the zona pellucida and neither their distribution nor accessibility appears to be affected by known biochemical and functional changes that the zona undergoes during development. Although similar in molecular properties to other extracellular matrix proteins, ZP-2 is found uniquely in the ovary surrounding growing oocytes. Other matrix glycoproteins are also found in the ovary but not surrounding the oocytes and, furthermore, they appear to be immunologically distinct from ZP-2.

One of the major difficulties in investigating molecular mechanisms of development in early mammalian embryogenesis is the paucity of biological material. Each zona pellucida contains ~5 ng of protein (1). However, using only 80 μ g of particulate zona we have developed monoclonal antibodies specific to the zona pellucida. Each of the antibodies described has been shown to be monoclonal by three criteria: (a) each cell line was subcloned by limiting dilution at least twice; (b) each monoclonal antibody has only one subclass of immunoglobulin heavy chain; (c) each antibody has a single and unique light chain as detected on O'Farrell two-dimensional gels. In addition, binding data for each of the monoclonal antibodies to eggs could be fitted to a straight line when plotted according to Scatchard.

ZP-2, the major component of the zona pellucida (1, 25), also appears to be the major antigen. Each of the five monoclonal antibodies described in this paper binds to ZP-2 and the epitope analysis indicates the presence of at least three distinct antigenic determinants. Two of these antibodies also bind to ZP-3 suggesting that ZP-2 and ZP-3 share a common structural element. Neither antibody will bind to [³H]galactose labeled ZP-2 or ZP-3 after digestion of the individually purified zona proteins with pronase (manuscript in preparation). Thus, the monoclonal antibodies appear to recognize a protein rather than a carbohydrate structure common to ZP-2 and ZP-3. This raises the intriguing possibility that the two proteins are members of a single developmentally regulated gene family. The cross-reactivity of ZP-2 and ZP-3 has not previously been demonstrated. Greve et al. (12) produced a polyclonal antiserum to murine ZP-2 that was unable to immunoprecipitate ZP-3. We have recently isolated a cell line that produces antibodies specific for ZP-3. Neither the polyclonal antiserum nor any of the more than 25 monoclonal antibodies characterized to date appear to immunoprecipitate ZP-1.

Indirect immunofluorescence using monoclonal antibodies to three different antigenic determinants on ZP-2 indicate that this major constituent of the zona pellucida is present throughout the zona. Under high magnification the zona appears as a fibrous network not unlike that reported in studies using scanning electron microscopy (7, 16, 17, 22). During oogenesis the size of the oocyte increases dramatically. The presence of the zona pellucida has been observed in oocytes with a diameter of 30 μ m (26) and its production continues in oocytes as large as 70 μ m (3, 25). This growth represents at least a fivefold increase in the surface area of the oocyte. However, the encompassing zona pellucida is a metabolically stable structure with a long half-life (25). Thus, the fenestrated network that we observe in the zona of ovulated eggs (which no longer synthesize zona proteins) may indicate flexibility of the earliest laid down zona proteins to accommodate the tremendous oocyte growth.

Although an extracellular glycoprotein, our studies indicate that ZP-2 is unlike other previously described matrix glycoproteins either in tissue distribution or in antigenicity. Taking

advantage of this observation, we have injected ZP-2 specific monoclonal antibodies into female mice. Preliminary results indicate that the antibody localizes to the ovary in vivo and studies are underway to determine the effect of the monoclonal antibody on the known functions of the zona pellucida in early development.

We are grateful to Dr. Michael Edidin for the fluorescence photobleaching recovery data, and to Drs. Constance Oliver and Mark Willingham for expert advice on the fluorescence microscopy. We appreciate the critical reading of the manuscript by Drs. Alan N. Schechter and Jay Berzofsky, and the expert typing of Ms. Dorothy Stewart.

Received and accepted for publication 11 October 1983.

REFERENCES

- Bleil, J. D., and P. M. Wassarman. 1980. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev. Biol.* 76:185-202.
- Bleil, J. D., and P. M. Wassarman. 1980. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell.* 20:873-882.
- Bleil, J. D., and P. M. Wassarman. 1980. Synthesis of zona pellucida proteins by denuded and follicle-enclosed mouse oocytes during culture *in vitro*. *Proc. Natl. Acad. Sci. USA.* 77:1029-1033.
- Bleil, J. D., C. F. Beall, and P. M. Wassarman. 1981. Mammalian sperm-egg interaction: fertilization of mouse eggs triggers modification of the major zona pellucida glycoprotein, ZP-2. *Dev. Biol.* 86:189-197.
- Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ¹²⁵I-containing acylating agent. *Biochem. J.* 133:529-539.
- Carlin, B., R. Jaffe, B. Bender, and A. E. Chung. 1981. Entactin, a novel basal lamina-associated sulfated glycoprotein. *J. Biol. Chem.* 256:5209-5214.
- Dudkiewicz, A. B., C. A. Shivers, and W. L. Williams. 1976. Ultrastructure of the hamster treated with zona precipitating antibody. *Biol. Reprod.* 14:174-185.
- Dunbar, B. S., N. J. Wardrip, and J. L. Hedrick. 1980. Isolation, physicochemical properties, and macromolecular composition of zona pellucida from porcine oocytes. *Biochemistry.* 19:356-365.
- East, I. J., J. G. R. Hurrell, P. E. E. Todd, and S. J. Leach. 1982. Antigenic specificity of monoclonal antibodies to human myoglobin. *J. Biol. Chem.* 257:3199-3202.
- Edidin, M., and T. Wei. 1982. Lateral diffusion of H-2 antigens on mouse fibroblasts. *J. Cell Biol.* 95:458-462.
- Eppig, J. J. 1977. Mouse oocyte development *in vitro* with various culture systems. *Dev. Biol.* 60:371-388.
- Greve, J. M., G. S. Salzmann, R. J. Roller, and P. M. Wassarman. 1982. Biosynthesis of the major zona pellucida glycoprotein secreted by oocytes during mammalian oogenesis. *Cell.* 31:749-759.
- Hurrell, J. G. R., N. A. Nicola, W. J. Broughton, M. J. Dilworth, E. Minasian, and S. J. Leach. 1976. Comparative structural and immunochemical properties of leghaemoglobins. *Eur. J. Biochem.* 66:389-399.
- Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.)* 256:495-497.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Longo, F. J. 1981. Changes in the zonae pellucidae and plasmalemmae of aging mouse eggs. *Biol. Reprod.* 25:399-411.
- Motta, P., and J. Van Blerkom. 1975. A scanning electron microscopic study of the luteo-follicular complex II. Events leading to ovulation. *Am. J. Anat.* 143:241-264.
- Nicolson, G. L., R. Yanagimachi, and H. Yanagimachi. 1975. Ultrastructural localization of lectin-binding sites on the zonae pellucidae and plasma membranes of mammalian eggs. *J. Cell Biol.* 66:263-274.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133-1142.
- Oi, V. T., and L. A. Herzenberg. 1980. Immunoglobulin-producing hybrid cell lines. In *Selected Methods in Cellular Immunology*. B. B. Mishell, and S. M. Shiigi, editors. W. H. Freeman and Co., San Francisco. 351-372.
- Phillips, D. M., and R. Shalgi. 1980. Surface architecture of the mouse and hamster zona pellucida and oocyte. *J. Ultrastruct. Res.* 72:1-12.
- Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Annu. NY Acad. Sci.* 51:660-672.
- Shimizu, S., M. Ito, and J. Dean. 1982. Glycoproteins of mouse zona pellucida: analysis of their reactivity to lectins. *Biochem. Biophys. Res. Commun.* 109:449-454.
- Shimizu, S., M. Tsuji, and J. Dean. 1983. *In vitro* biosynthesis of three sulfated glycoproteins of murine zonae pellucida by oocytes grown in follicle culture. *J. Biol. Chem.* 258:5858-5863.
- Wassarman, P. M., and W. J. Josefowicz. 1978. Oocytes development in the mouse: an ultrastructural comparison of oocytes isolated at various stages of growth and meiotic competence. *J. Morphol.* 156:209-236.
- Wolf, D. E., and M. Edidin. 1981. Diffusion and mobility in surface membranes. In *Techniques in Cellular Physiology*. P. F. Baker, editor. Elsevier/North-Holland Biomedical Press, Amsterdam.
- Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 50:761-799.
- Yanagimachi, R. 1981. Mechanisms of fertilization in mammals. L. Mastroianni Jr., and J. D. Biggers, editors. *Fertilization and Embryonic Development in vitro*. Plenum Press, New York. 81-182.