Generation and characterization of site-directed antisera against an amino-terminal segment of a 55 kDa sperm adhesive glycoprotein from zona pellucida of pig oocytes

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ZP3 α , a 55 kDa zona pellucida glycoprotein, mediates attachment of boar spermatozoa to zona-encased oocytes. In the study reported here, site-directed anti-peptide antibodies were used to probe the topography and biological activity of the amino-terminal domain of this sperm adhesive zona molecule. A synthetic peptide (α 8–18) corresponding to an amino-terminal sequence of ZP3 α (residues 8–18) was coupled to keyhole limpet haemocyanin (KLH) using either *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester or glutaraldehyde. Male rabbits were immunized with peptide–KLH conjugates using Freund's adjuvant, and antibody production was monitored by ELISA. High-titred anti-peptide sera were obtained and further characterized. Western blotting experiments using deglycosylated ZP3 α and ZP3 β core proteins demonstrated reactivity of anti-peptide sera with homologous protein. Preincubation of antisera with cognate peptide blocked binding to ZP3 α thus confirming site-specific binding of antibodies to the targeted sequence in the intact protein. Anti-KLH- α 8–18 sera reacted poorly with fully glycosylated ZP3 α , but ZP3 α immunoreactivity was markedly enhanced by digestion of polylactosamines with endo- β -galactosidase, chemical deglycosylation or protein unfolding. Finally, zona-encased pig oocytes were preincubated with the anti-peptide sera, washed and exposed to boar spermatozoa. Pretreatment with anti-KLH- α 8–18 sera significantly diminished attachment of boar spermatozoa to zonae. These studies demonstrate that the amino-terminal domain of pig oocyte zona protein, ZP3 α , is partially occluded, in part by carbohydrate, and that site-directed anti-peptide sera targeted to this portion of the molecule can interfere with sperm–zona attachment *in vitro*.

Introduction

The mammalian egg is surrounded by an extracellular envelope, the zona pellucida, which comprises acidic glycoproteins, the synthesis and secretion of which are developmentally programmed during oocyte growth and follicular development (Sacco *et al.*, 1981; Wassarman *et al.*, 1989). The zona represents the site of initial sperm–egg contact and specific zona molecules participate in crucial gamete recognition and activation events in the fertilization process. These events include, but are not limited to, binding of acrosome-intact (Bleil and Wassarman, 1980) and acrosome-reacted spermatozoa (Bleil *et al.*, 1988), induction of the sperm acrosome reaction (Bleil and Wassarman, 1983) and establishment of a secondary block to polyspermy (Wassarman, 1987).

The zona pellucida of pig oocytes comprises three biochemically and immunologically distinct glycoproteins (Hedrick and Wardrip, 1987; Yurewicz *et al.*, 1987) which, adopting the terminology in use in our laboratory, are referred to as ZP1 (82 kDa), ZP3 α (55 kDa; core peptide = 37 kDa) and ZP3 β (55 kDa; core peptide = 32 kDa). Several biochemical

characteristics of ZP3 α and ZP3 β have been reported. Each is extensively glycosylated (30% carbohydrate, w/w) with a heterogeneous population of sugar chains present on asparagine as well as serine/threonine residues (Hedrick and Wardrip, 1986; Yurewicz *et al.*, 1987; Nakano *et al.*, 1990; Yurewicz *et al.*, 1991; Noguchi *et al.*, 1992). Sequence analysis of *N*-terminal and *O*-glycosylated domains has been accomplished (Yurewicz *et al.*, 1987; Yurewicz *et al.*, 1992a) and demonstrates that ZP3 α and ZP3 β of pigs are homologous to rabbit rc55 (Schwoebel *et al.*, 1991) and mouse ZP3 (Ringuette *et al.*, 1988) zona proteins, respectively.

ZP3 α has been implicated as a zona molecule mediating binding of boar spermatozoa to the pig zona pellucida. Preincubation of boar spermatozoa with purified ZP3 α inhibits subsequent sperm attachment of pig zonae (Sacco *et al.*, 1989), and biotin-labelled ZP3 α binds to the anterior head of capacitated boar spermatozoa and to isolated boar sperm membrane vesicles in a dose-dependent and saturable manner (Yurewicz *et al.*, 1990). In complementary experiments, preincubation of zona-encased oocytes with anti-ZP3 α sera prevented subsequent attachment of boar spermatozoa (Sacco *et al.*, 1989), presumably a result of masking critical ZP3 α domains that are necessary for sperm binding. In addition, antisera raised against deglycosylated

ZP3 α core protein have been shown to inhibit sperm–zona attachment using human gametes (Henderson *et al.*, 1987). ZP3 α may therefore represent a promising target immunogen for development of an antifertility vaccine.

Immunization of female mice with a synthetic ZP3 peptide–carrier conjugate has been shown to elicit antibodies with contraceptive effects *in vivo* (Millar *et al.*, 1989). Such an approach should therefore be applicable to zona macromolecules of other species for which full or partial amino acid sequence information is available. Automated Edman degradation has defined the amino-terminal sequence of pig zona protein ZP3 α (Yurewicz *et al.*, 1987). In the present study, a synthetic peptide was used to generate site-directed antisera to the corresponding domain of this glycoprotein.

Materials and Methods

Chemicals

Custom synthetic peptides, selected from regions of maximum hydrophilicity (Hopp and Woods, 1981; Black and Glorioso, 1986) within amino acid sequences previously determined by Edman degradation of purified ZP3 α and ZP3 β (Yurewicz *et al.*, 1987), were obtained from Peninsula Laboratories (Belmont, CA). NH₂–Cys–Asp–Ala–Val–Pro–Val–Trp–Asp–Arg–Leu–Pro–COOH (peptide α 8–18) corresponds to residues 8–18 of ZP3 α and NH₂–Asp–Glu–Gly–Gln–Arg–Leu–Arg–Pro–Ile–Lys–Pro–(Cys)–COOH (peptide β 1–11), used as a control, corresponds to residues 1–11 of ZP3 β . Keyhole limpet haemocyanin (KLH), *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), glutaraldehyde, BSA and alkaline phosphatase conjugated goat anti-rabbit IgG were obtained from Sigma Chemical Co. (St Louis, MO); biotin-labelled goat anti-rabbit IgG, normal goat serum and alkaline phosphatase conjugated avidin were from Jackson ImmunoResearch Laboratories (West Grove, PA). Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Rockville Centre, NY).

Seven zona antigen preparations, each prepared as described previously (Yurewicz *et al.*, 1987), were used in the present study: ZP3 (purified preparation consisting of ZP3 α and ZP3 β); ZP3/EBGD (endo- β -galactosidase digested ZP3, consists of polylactosamine-deficient ZP3 α and ZP3 β); ZP3 α /EBGD [purified endo- β -galactosidase digested ZP3 α (46 kDa)]; ZP3/DG (chemically deglycosylated ZP3; consists of ZP3 α and ZP3 β core proteins); ZP3 α /DG (deglycosylated ZP3 α (37 kDa)); ZP3 β /DG (deglycosylated ZP3 β (32 kDa)); and ZP3/RCM (consists of reduced and carboxamidomethylated ZP3 α and ZP3 β).

Preparation of peptide–carrier conjugates

The peptide–carrier conjugate KLH– α 8–18 (MBS) was prepared by coupling peptide α 8–18 to MBS-activated KLH through peptide cysteine residues as described by Green *et al.* (1982). Briefly, 10 mg KLH in 0.92 ml of 10 mmol sodium phosphate l⁻¹, pH 7.2, was reacted (30 min, room temperature) with 1.2 mg MBS dissolved in 0.18 ml dimethylformamide. Excess MBS was removed by gel filtration at 4°C on a column of Sephadex G-25 equilibrated with 50 mmol sodium phosphate l⁻¹, pH 6.0. Peak fractions of KLH–MBS (1 ml total volume) were

coupled (3 h, room temperature) with 5 mg peptide dissolved in 1 ml of 250 mmol sodium phosphate l⁻¹, pH 7.2.

Preparation of the peptide–carrier conjugate KLH– α 8–18(GA) was accomplished by coupling peptide amino groups to KLH using glutaraldehyde according to Goldsmith *et al.* (1987). Briefly, 3 mg of peptide was incubated (24 h, room temperature) with 10 mg KLH in 1.5 ml 100 mmol sodium phosphate l⁻¹, pH 7.0, containing 8.3 mmol glutaraldehyde l⁻¹.

Production of polyclonal anti-peptide antibodies

Four male New Zealand white rabbits were immunized with the peptide–KLH conjugates [two with KLH– α 8–18(MBS) and two with KLH– α 8–18(GA)] using a multisite, intradermal injection protocol (Vaitukaitis *et al.*, 1971). After an initial injection of 0.17 mg of peptide–KLH conjugate emulsified in Freund's complete adjuvant, rabbits received two additional injections at weekly intervals consisting of identical doses prepared in Freund's incomplete adjuvant. Each animal was injected at day 108 after initial immunization with 0.5 mg of conjugate mixed with Freund's incomplete adjuvant. Total conjugate injected per rabbit was 1.0 mg. Sera were collected at periodic intervals beginning at 56 days after the initial injection and stored at –20°C.

ELISA

Reactivity of antisera with synthetic peptides as well as with various zona antigens was tested by ELISA performed in 96-well polystyrene microtitre plates. Wells were coated overnight at 4°C with duplicate or triplicate 50 ng aliquots of peptide or zona antigen dissolved in 50 μ l of 0.1 mol sodium carbonate l⁻¹, pH 9.6. After washing three times with high salt Tris (HST: 20 mmol Tris l⁻¹, 500 mmol NaCl l⁻¹, pH 7.5) containing 0.02% Tween-20 (HST/T), wells were blocked by incubation (1 h) with 200 μ l of HST containing 1% BSA. After washing with HST/T, wells were incubated for 2 h with 100 μ l primary antiserum diluted with HST/T. After additional washes in HST/T, 200 μ l of a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG was added. After a 2 h incubation and washing, each well received 200 μ l of 1 mg *p*-nitrophenyl-phosphate ml⁻¹ dissolved in 50 mmol sodium carbonate l⁻¹, pH 9.6, containing 1 mmol MgCl₂ l⁻¹. After 1 h at room temperature, the absorbance was determined at 405 nm in a manual microtitre plate reader (Cambridge Technology, Watertown, MA). All results were corrected for nonspecific binding by subtracting absorbance values obtained on duplicate microtitre plates not coated with antigens.

Electrophoresis and immunoblotting

SDS-PAGE of native and deglycosylated zona antigens was performed under nonreducing conditions on 10% polyacrylamide gels according to Laemmli (1970). Electrophoretic transfer of proteins to nitrocellulose (western transfer) was performed at 100 V for 1 h using the buffer system of Towbin *et al.* (1979). Visualization of transferred proteins on nitrocellulose was accomplished with Aurodyne (Janssen Pharmaceutica, Piscataway, NJ) according to the manufacturer's instructions.

Western blots destined for immunostaining were blocked overnight in HST and 5% BSA and subsequently washed with HST and 0.1% BSA. After incubation (2 h) with primary antiserum diluted in HST in 0.1% BSA containing 1% goat serum, the blots were developed by sequential incubations with biotin-labelled goat anti-rabbit IgG ($1.0 \mu\text{g ml}^{-1}$, 2 h) and avidin-conjugated alkaline phosphatase ($0.8 \mu\text{g ml}^{-1}$, 2 h). Colour development was achieved using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates.

Biotinylated protein standards were prepared by incubation of a mixture of reduced and carboxamidomethylated proteins (phosphorylase (94 kDa); BSA (68 kDa); glutamate dehydrogenase (54 kDa); glyceraldehyde-3-phosphate dehydrogenase (37 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (21 kDa); horse myoglobin (17 kDa); and aprotinin (6.5 kDa)) in 100 mmol sodium carbonate l^{-1} , pH 8.0, containing $20 \mu\text{g}$ D-biotin-N-hydroxysuccinimide ester ml^{-1} for 6 h at room temperature. The solution was then stored at -20°C and aliquots ($4 \mu\text{l}$) were used directly for calibration of western blots.

Sperm-zona attachment studies

Effects of antibody pretreatment of pig zonae on attachment of boar spermatozoa were measured using procedures previously described (Sacco *et al.*, 1984). Boar semen (Swine Genetics International, Cambridge, IA) were centrifuged at $800g$ for 10 min, and the seminal fluid was discarded. The spermatozoa were washed twice in 5 ml of culture medium (Peterson *et al.*, 1980), incubated at 37°C in 5% CO_2 in air in 4 ml medium for 30–60 min and then the number and motility of spermatozoa were assessed.

Cumulus-free zona-encased pig oocytes (10–15 per treatment group), prepared manually from minced ovaries, were preincubated in $50 \mu\text{l}$ of either preimmune or anti-peptide sera, used neat, for 20–30 min at room temperature. After washing in three changes of PBS, the oocytes were transferred to $40 \mu\text{l}$ of culture medium under oil (British Drug House Light Liquid Paraffin, Gallard-Schlesinger, Carle Place, NY) and 100 000 motile spermatozoa were added. Gametes were coincubated for 60–90 min at 37°C in 5% CO_2 in air. Oocytes were then gently washed once in fresh culture media, transferred to a microscope slide and viewed by bright field or phase contrast microscopy.

Statistical analysis

The number of spermatozoa bound to the zona in a single plane of focus was determined, and the average number of bound spermatozoa per oocyte was calculated for each treatment group. Results are presented as means $\pm$ SEM. Data were analysed by one-way analysis of variance followed by the Student–Newman–Keuls multiple range test. Differences were considered significant when $P < 0.05$.

Results

Time course of anti-peptide immune response

Antibody concentrations of sera collected between 56 and 161 days after immunization were monitored by ELISA using

Fig. 1. Inhibition of antisera binding to ZP3 α by cognate peptide. Microtitre plates coated with ZP3 α /EBGD were reacted with (a) anti-KLH- α 8-18(GA) serum and (b) anti-KLH- α 8-18(MBS) serum pre-incubated in the presence and absence of indicated concentrations of free peptide, either (●) α 8-18 or (▽) β 1-11. Absorbance values in the absence of competing peptide were >0.5 . KLH: keyhole limpet haemocyanin.

unconjugated α 8-18 peptide and homologous protein (ZP3 α /EBGD) as immobilized antigens. Maximum immune responses were obtained within 100 days and were not substantially increased after the booster immunizations (108 days). Immuno-reactivity against ZP3 α /EBGD was approximately 10–20% of that obtained with α 8-18 peptide and decreased 70–100 days after immunization. Further studies therefore used antisera from the second collection of blood samples (63 days) from the rabbit of each pair yielding the better ELISA result toward ZP3 α /EBGD antigen. Preimmune sera did not bind α 8-18 or ZP3 α /EBGD.

Specificity of anti-peptide antibodies for cognate peptide

The titres of anti-peptide antibodies were determined in ELISA by reacting serial serum dilutions with immobilized α 8-18. Antibody titres, defined as the serum dilution yielding one-half the maximum binding level, were similar: 1:30 000 for anti-KLH- α 8-18(GA) and 1:25 000 for anti-KLH- α 8-18(MBS). No crossreaction of anti- α 8-18 sera with a heterologous zona peptide, β 1-11, was observed.

The site specificity of the anti-KLH- α 8-18 sera was confirmed for the corresponding sequence in ZP3 α /EBGD by preincubating antisera with α 8-18 prior to reaction against the immobilized glycoprotein. Dose-dependent inhibition of antibody binding to ZP3 α /EBGD by homologous (α 8-18), but not heterologous (β 1-11), peptide was observed (Fig. 1).

Reactivity of anti-peptide sera with native versus modified ZP3 α preparations

ELISA techniques demonstrated a marked influence of tertiary structure and glycosylation on the accessibility of the

Fig. 2. Immunoreactivity of native and modified zona glycoproteins with site-specific antisera. The recognition of native, ZP3, endo- β -galactosidase digested ZP3 (ZP3/EBGD), trifluoromethanesulfonic acid treated ZP3 (ZP3/DG) and reduced, carboxamidomethylated ZP3 (ZP3/RCM) by 1:400 dilutions of (□) anti-KLH- α 8-18(GA) serum and (▨) anti-KLH- α 8-18(MBS) serum was assessed by ELISA.

peptide epitope of ZP3 α . Antibody binding was determined by ELISA using four different ZP3 preparations as ZP3 α -containing target antigens: (1) native ZP3; (2) ZP3/EBGD (approximately 30% deglycosylation via endo- β -galactosidase digestion of polylactosamines); (3) ZP3/DG (>92% deglycosylation via treatment with trifluoromethanesulfonic acid); and (4) ZP3/RCM (disruption of tertiary structure via reduction and alkylation of disulfide bonds) (Fig. 2). Binding of anti-KLH- α 8-18 sera to immobilized ZP3 was only marginally detectable. Perturbation of native structure by deglycosylation or disruption of disulfide bonds, however, markedly enhanced binding of anti-KLH- α 8-18 sera as evidenced by increased immunoreactivity of modified ZP3 preparations in the order of ZP3/RCM > ZP3/DG > ZP3/EBGD > ZP3.

Immunoblotting experiments

Specificity of anti-KLH- α 8-18 sera for homologous protein was also investigated by immunoblotting experiments. ZP3 α /DG (37 kDa) and ZP3 β /DG (32 kDa) were electrophoresed on nonreducing SDS-polyacrylamide gels and western blots were probed with anti-KLH- α 8-18 sera (Fig. 3). ZP3 and ZP3/RCM were also included in the experiment to confirm the differences in their relative immunoreactivities indicated by ELISA. Protein staining of the nitrocellulose blots revealed efficient transfer of all four antigens. Immunostaining with anti-KLH- α 8-18(MBS) serum demonstrated strong immunoreactivity with ZP3/RCM and ZP3 α /DG, minor crossreactivity with ZP3 β /DG and no detectable binding to ZP3. Identical results were obtained with antisera prepared against glutaraldehyde cross-linked conjugates (not shown).

Contraceptive activity of antipeptide sera

The ability of antisera to the synthetic ZP3 α peptide to interfere with sperm-zona attachment was evaluated *in vitro* using pig gametes. Zona-encased pig oocytes were pretreated

with preimmune sera or antisera, washed and incubated with capacitated boar spermatozoa. Pretreatment with antisera to the synthetic α 8-18 peptide-KLH conjugates had a significant effect ($P < 0.05$) on sperm-zona attachment when compared with the control group consisting of oocytes exposed to preimmune sera (Table 1).

Discussion

In this report a synthetic peptide has been successfully used as an immunogen to generate antisera directed against a defined amino-terminal segment of the zona pellucida glycoprotein ZP3 α of the pig oocyte. Although undetermined at the time these studies were initiated, recent cDNA cloning experiments have revealed an 82% identity between the α 8-18 peptide (CDAVPVWDRLP) and residues 123-133 (CDSVPVQDRLP) of a 55 kDa rabbit zona protein (Schwoebel *et al.*, 1991). The homology between peptide α 8-18 and a segment of the rabbit 55 kDa protein would not be expected to influence antibody production in these studies since immunizations were performed in male rabbits.

Two methods were used for covalent attachment of α 8-18 synthetic peptide to the KLH carrier protein: via peptide amino groups using glutaraldehyde or via an endogenous peptide cysteine residue using MBS reagent. Both coupling methods yielded immunogens that elicited antipeptide sera with qualitatively similar binding specificities and biological activity. Quantitatively, the KLH- α 8-18(GA) and KLH- α 8-18(MBS) conjugates also elicited similar titres. An advantage of one coupling method over the other was therefore not apparent in this study.

The site specificity of the anti- α 8-18 sera was confirmed by western blotting and competition-type ELISA experiments. Anti-KLH- α 8-18 sera immunostained the respective deglycosylated core protein, ZP3 α /DG, on western blots. However, the slight but discernible crossreactivity with ZP3 β /DG was unexpected and indicates a potential sequential or conformational epitope on ZP3 β that interacts with anti-KLH- α 8-18 sera with low affinity. Importantly, ELISA experiments demonstrated that binding of anti-KLH- α 8-18 sera to ZP3 α /EBGD was quantitatively blocked by preincubation of antisera with soluble cognate peptide under conditions where an unrelated zona peptide was without effect. Such results confirm that the anti-KLH- α 8-18 sera are in fact targeted to the selected peptide epitope and strongly argue against crossreactivity of anti-KLH antibodies, also present in the antisera, with ZP3 α .

Both ELISA and western blotting experiments demonstrated that the amino-terminal domain of purified ZP3 α molecules is poorly accessible to the site-directed peptide antibodies. Whereas ZP3 reacts only weakly with anti-KLH- α 8-18 sera, its immunoreactivity was significantly enhanced by removal of carbohydrate or perturbation of protein tertiary structure. Both partial enzymatic deglycosylation of ZP3 α with endo- β -galactosidase (approximately 33% sugar removal) and chemical deglycosylation (>92% sugar removal) enhanced immunoreactivity of ZP3 α with anti-KLH- α 8-18 sera. The former result implies that polylactosamines, which are a predominant component of ZP3 α N- and O-linked oligosaccharides (Yurewicz *et al.*, 1991; Noguchi *et al.*, 1992), contribute to the steric

Fig. 3. Immunoblotting with antipeptide serum. SDS-PAGE was performed using the following samples: lane a, biotin-labelled protein markers (molecular masses in kDa indicated on the far left); lane b, ZP3 (500 ng); lane c, ZP3/RCM (500 ng); lane d, ZP3 α /DG (200 ng); lane e, ZP3 β /DG (200 ng). After western transfer, the blots were processed as follows: left, protein staining with Aurodyne; right, immunostaining with anti-KLH- α 8-18(MBS) serum (1:1000 dilution).

Table 1. Effects of pretreatment of pig oocytes with antisera to synthetic α 8-18 peptide on attachment of boar spermatozoa

Treatment serum	Number of oocytes	Mean number of attached spermatozoa ($\pm$ SEM)
Preimmune	86	25.3 $\pm$ 0.8 ^a
Anti-KLH- α 8-18(GA)	166	14.2 $\pm$ 0.8 ^b
Anti-KLH- α 8-18(MBS)	167	13.0 $\pm$ 0.8 ^b

Each serum was tested undiluted. Calculations are based on spermatozoa within a single plane of focus. Means identified by different superscripts are significantly different ($P < 0.05$) when tested by analysis of variance.

KLH: keyhole limpet haemocyanin.

hindrance of the peptide epitope recognized by the site-directed antibodies. Glycopeptide sequencing indicates that the α 8-18 segment is not immediately adjacent to glycosylated asparagine or serine/threonine residues (Yurewicz *et al.*, 1992a, b). It is therefore noteworthy that reduction and alkylation of ZP3 α disulfide bonds in the presence of chaotropic agent, conditions conducive to unfolding of the polypeptide core, also results in enhanced immunoreactivity with anti-KLH- α 8-18 sera. ZP3 α has a high content of sulphhydryl residues (13–15 mol cysteine mol⁻¹ protein). Presumably, the protein backbone of ZP3 α is folded and cross-linked by disulfide bonds in such a manner that a sugar chain anchored at a glycosylation site remote from the amino terminus in the primary sequence is maintained in a position proximal to the α 8-18 epitope.

Preincubation of zona-encased oocytes with anti-KLH- α 8-18 sera resulted in diminished binding of boar spermatozoa *in*

vitro. This result provides strong evidence that the site-directed antibodies have access to the α 8-18 epitope on ZP3 α and can sterically hinder zona ligands, presumably carbohydrate chains (Yurewicz *et al.*, 1991; Noguchi *et al.*, 1992), recognized by boar spermatozoa. Since the α 8-18 epitope has limited accessibility to antibodies in purified ZP3 α preparation, as discussed above, such results suggest that the amino-terminal domain of ZP3 α molecules in the intact zona pellucida is in a more open configuration. Presumably, the experimental conditions used for zona protein purification, i.e. heat solubilization of zonae and SDS treatment, lead to conformational changes in ZP3 α that result in reduced exposure of the α 8-18 epitope.

The inhibition of sperm binding observed with anti-KLH- α 8-18 sera, approximately 45–50% under the experimental conditions used, is not as great as previously observed with oocytes pretreated with anti-ZP3 α sera (Sacco *et al.*, 1989). This may reflect quantitative differences in antibody titres or number of antibodies bound to the zona. Alternatively, the anti-ZP3 α sera may contain antibodies that recognize carbohydrate epitopes and directly mask sperm binding sites, whereas the anti-KLH- α 8-18 sera presumably act by an indirect, and potentially less effective, steric inhibitory mechanism.

Although a quantitatively effective *in vitro* contraceptive effect was not observed with anti-KLH- α 8-18 sera at the level of sperm–zona attachment, effects on zona penetration or fertilization were not assessed and cannot be excluded. The results of the study reported here therefore encourage a more systematic evaluation of synthetic ZP3 α peptides. Once the primary sequence of ZP3 α has been elucidated, work which is currently in progress, antisera directed to additional internal and carboxy-terminal ZP3 α peptides can be produced and screened for ability to inhibit sperm–zona interactions. Production of anti-peptide sera with potent contraceptive efficacy *in vitro* would

not only serve to map the location of putative ZP3 α sperm-binding domains more precisely but, in view of the immunological similarity between zonae of pig and other mammalian species, would also encourage further evaluation of synthetic pig zona peptides as immunogens for antifertility vaccine development (Dean *et al.*, 1991).

The authors gratefully acknowledge C. Brusca for expert technical assistance. This research was supported in part by NIH grants HD 10189 and HD 23163.

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