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Diversity of antigenic determinants of porcine zona pellucida revealed by monoclonal antibodies

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Abstract. Monoclonal antibodies derived from ten hybrid cell clones, generated against porcine zona pellucida gave strong immunofluorescence with zona but the pattern varied from patchy, thin rim to heavy precipitation type of rim. Five of the 6 monoclonals studied prevented the binding of the porcine epididymal sperm to homologous oocytes *in vitro,* whereas the sixth one was partially effective. All of the 6 monoclonale of this batch inhibited the lysis of zonae by proteolytic enzymes even at dilutions up to 1×10^{-3}. Three of the four monoclonals prepared in a subsequent batch gave strong immunofluorescent reactions and had high titres as determined by enzyme immunoassay. These monoclonals did not, however, protect the zonae against lysis by proteolytic enzymes. These properties are suggestive of the heterogeneity of the antigenic determinants in zona and emphasize the employment of appropriate bioassays for screening and selection of bioeffective antibodies.

Keywords. Prevention of sperm binding; inhibition of proteolytic lysis of zona; immunofluorescence; enzyme immunoassay.

Introduction

Mammalian oocyte is covered by an acellular membrane, the zona pellucida (ZP) which is composed of glycoproteins. Spermatozoa must bind to the exterior of ZP and then penetrate through it to bind to the plasma membrane of the egg (Austin and Braden, 1956; Yanagimachi, 1977; Bedford and Cooper, 1978; Hartmann, 1983). The dissolution of ZP by a zona lysin (which is believed to be a proteolytic enzyme) is considered to be an important pre-requisite to the implantation of the blastocyst (Dickmann, 1969; Mclaren 1969, 1970; Denker 1980; Saling, 1981). Antibodies to ZP cause several changes in the zona such as precipitation, apparent in dark phase microscopy (Oikawa and Yanagimachi, 1975; Gwatkin, 1979; Tsunoda and Chang, 1976; Sacco and Palm, 1977; Shivers and Dunbar, 1977), prevention of sperm binding and penetration (Shivers *et al.,* 1972; Garavoguo *et al.,* 1974; Jilek and Pavlok, 1975; Yanagimachi *et al.,* 1976). The antibodies also prevent zona lysis by proteolytic enzymes (Ownby and Shivers, 1972; Garavaguo *et al.,* 1974; Tsunoda and Chang 1976). These properties

Abbreviations used: EIA, Enzyme immunoassay; ZP, zona pellucida; MoAb, monoclonal antibodies; DMEM, Dulbecco's modified Eagle's medium, BSA, bovine serum albumin; PBS, 0·01 M phosphate buffered-saline pH 7·2; PA-HRP, protein A-horse radish peroxidase; FITC, fluorescein isothiocyanate.

presumably account for the block of fertility noticed in animals by active immunization (Mahi-Brown *et al.,* 1982).

A disadvantage of active immunization is the atresia of follicles noticed in rabbits immunized with cross-reactive porcine zonae (Wood *et al.,* 1981). On the other hand passive immunization has been reported to be reversible, the block of fertility lasting for about 4–6 cycles in rodents following a single injection of the antibodies (Suman, 1980; Aitken and Richardson, 1980). Attempts have been made to raise monoclonal antibodies (MoAb) to pork zona pellucida which has antigens cross-reactive with the human zona. Isojima *et al.* (1984) reported the development of five clones and (Dunbar *et al.,* 1980) 6 clones against porcine zonae (Drell and Dunbar, 1984). We have communicated earlier successful development of six stabilized hybrid cell clones (Bamezai *et al.,* 1983). Four new clones have since been added to this library. Studies on the properties of these monoclonals indicate the presence on zonae of species restricted determinants besides those which are cross-reactive. These studies also point to epitopes against which antibodies give immunofluorescence but do not exercise the biologically important property of inhibition of zona lysis by proteolytic enzymes.

Materials and methods

Immunization and Hybridization

BALB/c mice were immunized subcutaneously with 0·5 ml of 1000 PZP in saline emulsified with equal amount of Freunds complete adjuvant at 4 sites, 3 booster injections with 500 PZP in 0·5 ml saline emulsified with equal amount of Leiras basic adjuvant (Leiras, Pharmaceutical Plant, Turku, Finland) were given at weekly intervals. Mice were sacrificed 3 days after the last injection given intraveneously and splenocytes were fused with SP2/0 azaguanine resistant cells using 0·5 ml of 50 % polyethylene glycol 1500 (Serva). The fused cells were suspended in 25 ml of Dulbecco's modified Eagle's medium (DMEM) (GIBCO) constituted with hypoxanthin, aminopterin thymidine (Sigma) and gentamycin 20 μg/ml. The cells were distributed in 96 well tissue culture plates (Flow Laboratories) which on previous day was layered with feeder cells derived from peritoneum of inbred BALB/c mice at 5×10^4 cells per well. Cells were grown at 37°C in the atmosphere of 5 % CO_2 and 95% air.

Screening of the hybrid clones

After 15 days of fusion, growth was seen in 90 % of the wells, the supernatants of the wells were checked for antibody production by enzyme immunoassay (EIA). The microtitre plates (Falcon) were coated with 100 μ1 of dissolved zonae (protein concentration 6·6 μg/ml) by drying at 37°C or room temperature (25°C). PZP were dissolved at pH 9·4 at 60°C for 60 min (Dunbar *et al.,* 1980). Non-specific sites of the wells were saturated with 2 % bovine serum albumin (BSA) in 0·01 M phosphate buffered saline pH 7·2 for 1 h at 37°C, and incubated with 100 μ1 of supernatant from hybrid cell clones for 2 h at room temperature. This was followed by washing 6 times with 0·01M PBS containing 0·1 % BSA and incubation with 100 μ1 of protein A-horse radish peroxidase (PA-HRP) conjugate at 1:400 dilution for 2 h at room temperature.

After 6 washes the enzyme was quantitated by measuring the peroxidase bound to the solid phase by adding 100 μl of substrate and chromogen solution (0·1 % H_2O_2 and orthophenylene diamine 1 mg/ml in citrate buffer pH 5). Reaction was stopped by adding 50 μl of 5 N H_2SO_4 after 10 min. Absorbance was measured at 492 nm on Pye Unicam spectrophotometer after diluting the solution 5 times.

Cloning of hybrid cells and production of monoclonal antibody

The hybrid cell clones demonstrating antibody activity upto a dilution of 1:5 in EIA were propagated and subcloned by limiting the dilution. After subcloning twice, the cells were grown as ascites in peritoneum of inbred BALB/c mice. The mice were primed with 0·5 ml Pristane (Aldrich), 7–12 days before injection of 5×10^6-1×10^7 hybrid cells suspended in 1 ml of DMEM Medium under sterile conditions. Ascites was tapped with 18G needle, centrifuged at 1000 *g* for 5 min to render it free from cells and passed through Milipore membrane filter (Pore size 0·45 μm) and stored at –20°C.

Isotyping

The MoAb were coated on microtitre plates (Falcon) at 4°C overnight. The supernatant was removed and the non-specific sites saturated with 2 % BSA in 0.9 % saline for 2 h at room temperature. This was followed by incubation with specific anti-mouse immunoglobulin subclass antisera tagged to alkaline phosphatase for 2 h at room temperature. After washing 6 times with 0·9 % saline, 100 μl of substrate solution (*p*-nitrophenyl phosphate 1 mg/ml in ethanolamine buffer pH 9·8) was added. The reaction was terminated after 30 min by adding 100 μ1 of 1 N NaOH and absorbance was determined at 440 nm in Pye Unicam spectrophotometer after 5 fold dilution.

Characterization and reactivity of the MoAb

Immunofluorescence: This was checked by indirect immunofluorescent staining of Porcine oocyte. Acetone fixed oocytes were incubated with 50 μl of diluted ascites fluid (5 mg/ml) for 30 min at room temperature in humid chamber. This was followed by extensive washing with PBS 0·1 M, pH 7·4 and treatment with 50 μl of rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Dekopet) at 1:20 dilution, for 30min in humid chamber. The slides were washed several times with PBS then mounted in glycerol for examination in fluorescence microscope (Carl Zeiss).

Proteolytic lysis assay: Ten porcine oocytes per well were placed in microtitre plates and incubated with 100 μl of MoAb at the indicated dilution for 2h at room temperature followed by washing with PBS 0·01 M pH 7·2, 3 times, to remove the unbound antibodies. The zonae were treated with 100 μl of 0.3 % trypsin (Difco, 1:250 this preparation contains other proteolytic enzymes) made in 0.01 M PBS, pH 7·2. The time taken for dissolution of the zonae observed under dissection microscope was recorded.

Sperm binding assay: Porcine oocytes were taken in microtitre plates (10 oocytes/well) and were incubated with 100 μl of MoAb for 2 h at room temperature. Spermatozoa of porcine cauda epididymal origin were suspended in medium (BWW) and checked for

motility. The suspension was centrifuged at 900 *g* for 5 min and washed 3 times with the medium. The pallet was resuspended in the medium to which was added, human serum albumin (Sigma) at 3 mg/ml final concentration. Sperm were kept at 37°C for 45 min for capacitation. Oocytes with and without preincubation with MoAb's were washed with PBS and incubated with 100 μl of 1×10^7 sperm/ml in watch glass. The gam te suspension was overlayered with paraffin oil to prevent desscication. After 1 h sperm attachment was observed under phase contrast microscope. For sperm binding studies, oocytes were sucked in and out of a Pasteur pipette 4 times so as to detach the loosely adhered sperms. The oocytes were then transferred to a glass slide, covered with a drop of glycerol and cover slip, for microscopic observation.

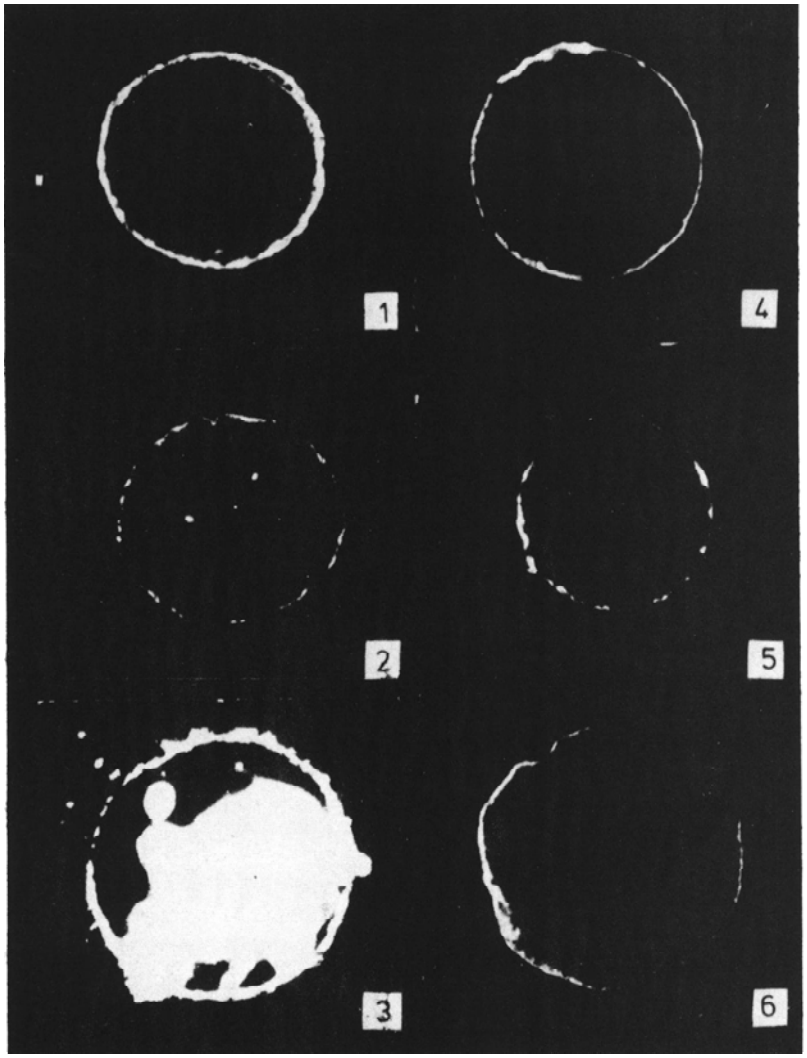

Figure 1. Reactivity of different monoclonals with porcine oocytes as visualized by immunofluorescence.

Results and discussion

The product of all hybrid cell clones gave immunofluorescence with zona layer around the porcine oocytes. However, the pattern was not identical, some monoclonal antibodies gave patchy reaction, others a thin rim and still others a heavy precipitation type of ring (figure 1). These observations suggest that the antigenic determinants recognized by these antibodies have a variable distribution in the zona. The complex nature of the components of zona pellucida is also indicated, which is in conformity with other physicochemical studies (Dunbar *et al.,* 1980).

Five monoclonale out of 6 studied prevented the binding of the homologous sperm to the porcine oocyte. Figure 2d is an illustrative example of the results obtained with the products of these hybrid cell clones. The monoclonals did not interfere completely with the attachment of the sperm (figure 2c) but the binding was inhibited. The sixth Mo Ab which was found inadequate to impede the binding of sperm gave the pattern of the type shown in figure 2e. The possibility, that the five MoAb which prevented sperm binding may be replicates of the same clone was excluded, not only on the grounds of extensive subcloning, but also because the isotype of these antibodies was of at least two types IgG_1 *k* and IgG_2 *k.* Three out of 10 MoAb's were not found to inhibit the lysis of zona by proteolytic enzymes inspite of the fact that they gave Strong reaction in EIA as

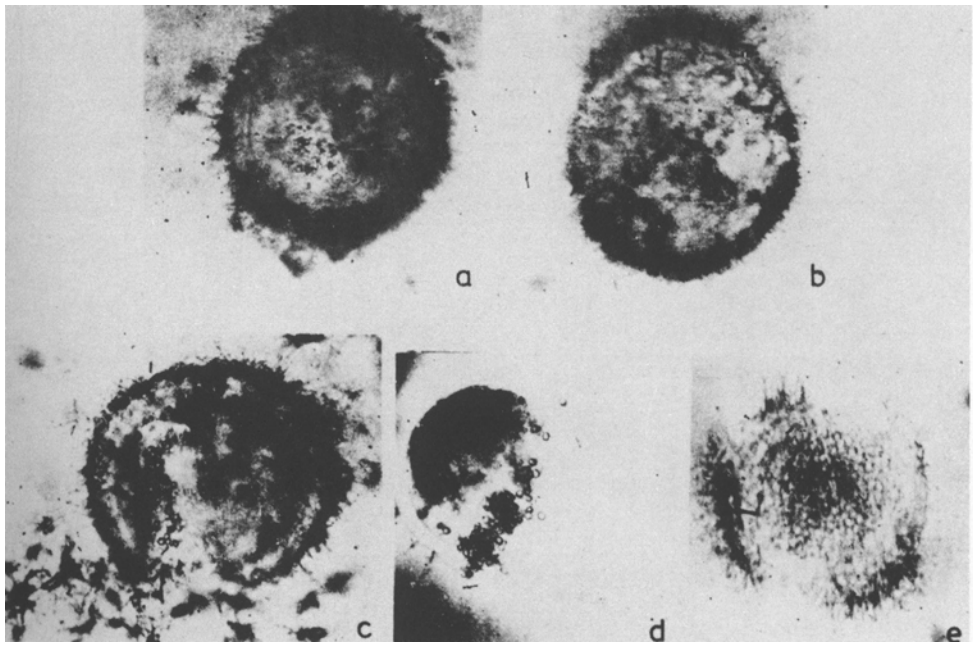

Figure 2. Influence of MoAb on attachment and binding of porcine sperm with homolog ous oocytes *in vitro* (a) is the attachment of sperm in the presence of irrelevant ascites fluid from Sp2/0, fusion partner myeloma cells; (b) sperm binding in the presence of Sp2/0 ascites; (c) sperm attachment; (d) sperm binding pattern in the presence of 5 of the 6 monoclonals in the medium; (e) sperm binding in the presence of the sixth clone G8 which was partially effective.

well as gave positive immunofluorescence. Tables 1 and 2 summarize these results.

From the above studies 3 types of immunological diversities are evident. An antibody may give clearly visible immunofluorescence on zonae, but the epitopes to which these are directed may not result in aggregation of the type necessary to be resistant to the action of the proteolytic enzymes. In order that an antibody is bioeffective, it should have one or both traits of impairing the binding of the sperm to the zonae, and according resistance to lysis of zona by proteolytic enzymes and it is not known which of these may be more important. The fact that one of the Mo Ab inhibited fairly well the lysis of zonae by impure trypsin but not adequately the sperm binding (figure 2) suggests that the two properties need not accrue from the same antigenic determinants. It was relevant to inquire whether the prevention of the sperm binding with antobodies is due to the MoAb directed against sperm, zona or determinants common to both gametes. Experiments were carried out to see whether sperm could absorb out the antibody. Data in figure 3 excludes this assumption. Hence, it can be suggested that these monoclonals generated to zonae do not share antigenic determinants of the sperm. Consequently, it can be postulated that none of these is an anti-idiotypic antibody. Selection of the hybrids for anti-idiotopes should be of interest for delineation and isolation of the sperm surface molecules interacting with zona.

The present studies point out to the possibility of developing hybrid cell clones

Table 1. Zona lysis by proteolytic enzymes.

Ascites (MoAb)	Dilution of Ascites	Duration of protection from lysis (min)
Buffer	—	14 ± 1
Sp2/0 (Fusion partner non-secreting myeloma cell line)	10^{-1}	15 ± 1
Anti-hCG MoAb (P_3W_{80})	10^{-1}	15 ± 1
A_2	$10^{-1}, 10^{-2}, 10^{-3}$	> 120
	10^{-4}	15 ± 1
A_6	$10^{-1}, 10^{-2}, 10^{-3}$	> 120
	10^{-4}	15 ± 1
C_{10}	$10^{-1}, 10^{-2}, 10^{-3}$	> 120
	10^{-4}	15 ± 1
G_8	$10^{-1}, 10^{-2}, 10^{-3}$	> 120
	10^{-4}	15 ± 1
H_9	$10^{-1}, 10^{-2}, 10^{-3}$	> 120
	10^{-4}	15 ± 1
D_{10}	$10^{-1}, 10^{-2}, 10^{-3}$	> 120
	10^{-4}	15 ± 1
C_3F_7	$10^{-1}, 10^{-2}, 10^{-3}$	> 120
	10^{-4}	15 ± 1
F_7D_{10}	10^{-1}	15 ± 1
G_6B_{10}	10^{-1}	15 ± 1
E_{12}	10^{-1}	15 ± 1

Table 2. Dissociation between the EIA titres and ability to prevent the lysis of zona by proteolytic enzymes.

MoAb	EIA titres of ascites at the dilution tested	Immuno-fluorescence	Titres upto which protective in zona lysis assay
A_2	1×10^{-5}	4+ (thick precipitin rim)	+ ve upto 10^{-3}
A_6	1×10^{-5}	3+ (thin rim)	–do–
C_{10}	–do–	4+ (Patchy)	–do–
G_8	–do–	3+ (thin rim)	–do–
H_9	–do–	3+ (thin rim)	–do–
D_{10}	–do–	3+ (thin rim)	–do–
C_3F_7	–do–	3+	–do–
D_7F_{10}	–do–	3+	not protective
G_6B_{10}	–do–	3+	not protective
E_{12}	–do–	3+	not protective

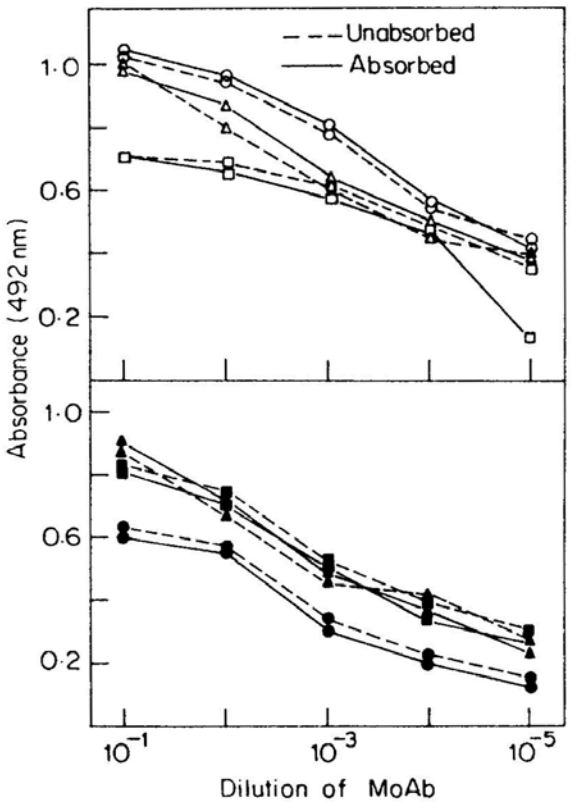

Figure 3. Reactivity of 6 monoclonal antibody after absorption two times with 1×10^6 porcine spermatozoa at room temperature for 1 h. (O) absorbed A_2; (---O--) unabsorbed A_2; (Δ) absorbed C_{10}; (--Δ--) unabsorbed C_{10}; (□) absorbed H_9; (--□--) unabsorbed H_9; (●) absorbed G_8; (--●--) unabsorbed G_8; (▲) absorbed A_6; (--▲--) unabsorbed A_6; (■) absorbed D_{10}; (-- ■---) unabsorbed D

against the zona antigens which secrete in large amount antibodies capable of preventing the binding of sperm to egg and of changing the structure of the zona in a manner so as to render it resistant to lysis by proteolytic enzymes. The present studies have been carried with porcine oocytes and homologous sperm. Antigenic cross-reactivity was reported between the pig and human zona (Sacco *et al.,* 1981). These antibodies were however, not been tested in the human system. All antibodies were devoid of cross-reaction with the mouse, rat and hamster zonae. Two of these clonal antibodies were observed by Dr. Mahi-Brown to be cross-reactive with canine zonae (personal communication). It will be of interest to see whether passive administration of these antibodies to female dogs can block fertility in these species. Given the antigenic diversity, the usual criteria of screening antibodies by immunofluorescence and EIA may not be adequate to assure antibodies of bioeffective potential.

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