Analysis of an epitope sequence recognized by a monoclonal antibody MAb-5H4 against a porcine zona pellucida glycoprotein (pZP4) that blocks fertilization

A. Hasegawa¹, N. Yamasaki¹, M. Inoue¹, K. Koyama^{1*} and S. Isojima²

¹Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan; and

²Advanced Fertility Center, Fuchu Hospital, Izumifuchu, Osaka, Japan

The zona pellucida glycoprotein that surrounds the mammalian oocyte has several target antigens that have potential use in the development of a contraceptive vaccine. In the present study, an epitope sequence recognized by a monoclonal antibody to the porcine zona pellucida glycoprotein ZP4 was determined. Three candidate peptides were synthesized, based on an epitope mapping by cDNA and an analysis of chain flexibility of porcine ZP4. Only one synthetic peptide, corresponding to amino acid positions 50–67, reacted with the monoclonal antibody; the other synthetic peptides, corresponding to positions 60–79 and 70–100, did not react. The reactive epitope was identified as CTYVLDPENL, corresponding to positions 50–59 of porcine ZP4. The peptide inhibited the reaction of the monoclonal antibody binding to native ZP4 in a dose-dependent manner. When the synthetic peptide 50–67 was used to immunize mice, the resultant antisera reacted not only with the synthetic peptide but also with native pig zona pellucida. In addition, anti-peptide 50–67 antibody inhibited porcine fertilization *in vitro*. It is thus concluded that the peptide identified as an epitope for the monoclonal antibody would be a promising candidate for the development of a contraceptive vaccine.

Introduction

Mammalian oocytes are surrounded by an extracellular matrix known as the zona pellucida, which is synthesized and secreted by oocytes (Wassarman, 1990; Wassarman and Mortillo, 1991; Liang and Dean, 1993a). The zona pellucida consists of several glycoproteins which play a role in gamete recognition, sperm activation and prevention of polyspermy. In mice, the zona pellucida comprises three glycoproteins, referred to as mZP1, mZP2 and mZP3. Mouse ZP3 has been examined intensively as a primary sperm receptor and as an inducer of the acrosome reaction in the process of fertilization. Recently, a domain-bearing sperm receptor function was identified for mZP3 by use of recombinant DNA technology (Kinlock *et al.*, 1995). Murine ZP2 serves as a secondary sperm receptor that retains the acrosome-reacted spermatozoa during penetration through the zona pellucida by the interaction of mZP2 with ligands on the spermatozoa (Bleil *et al.*, 1988). The third component mZP1 is a structural component that maintains the filamentous structure of the zona pellucida by interconnecting with mZP2 and mZP3 heterodimers (Wassarman and Mortillo, 1991). Pig zona pellucida is composed of three glycoprotein families, pZP1, pZP3 α and pZP3 β . pZP1 is divided into pZP2 and pZP4 by reduction of S–S bonds (Hedrick 1993; Hasegawa *et al.*, 1994). pZP3 α is a primary receptor for boar spermatozoa (Sacco *et al.*, 1989; Yurewicz *et al.*, 1993a).

Early studies showed that the zona pellucida from heterologous species evoke a strong immune reaction and that antisera to the zona pellucida can inhibit fertilization (Oikawa and Yanagimachi, 1975; Gwatkin *et al.*, 1977; Tsunoda and Chang, 1978; Sacco, 1979). On the basis of this species crossreactivity of zona antigens (Sacco, 1977; Drell and Dunbar, 1984; Isojima *et al.*, 1984), studies were undertaken on the development of contraceptive vaccines (Isahakia and Bambara, 1992; Aitken *et al.*, 1993; Sacco and Yurewicz, 1993; Naz *et al.*, 1995). However, animals immunized with zona pellucida components showed ovarian dysfunctions and the deletion of primordial follicles resulting in premature menopause (Sacco *et al.*, 1983; Skinner *et al.*, 1984; Mahi-Brown *et al.*, 1988; Hasegawa *et al.*, 1992). It is therefore necessary to segregate the epitope that inhibits the sperm–zona pellucida interaction from other epitopes that induce ovarian failure.

It has been shown that antibodies to pZP1 are most effective in blocking fertilization (Hedrick, 1993; Hasegawa *et al.*, 1988). Koyama *et al.* (1991) reported that a monoclonal antibody against pZP4 (MAb-5H4) that corresponds to the amino-terminal region of pZP1 could inhibit the binding of human spermatozoa to homologous zona pellucida. Since MAb-5H4 reacts not only with pig zona pellucida but also with those from many other species, a model could be designed for human contraception. Recently, several new technologies have been developed for the research and development of vaccines, such as mapping of the immunogenic domains by monoclonal antibodies (Millar *et al.*, 1989; Yurewicz *et al.*, 1993b; Gupta

*Correspondence.

Received 27 April 1995.

et al., 1994). In the study reported here, we attempted to determine the epitope of MAb-5H4 using epitope mapping and protein flexibility analysis of pZP4. It was found that the epitope consists of a sequence of ten amino acids CTYVLD-PENL and that antiserum raised to the synthetic peptide containing the ten amino acid peptide recognizes intact pig zona pellucida. The fertilization blocking effect of the antiserum is also described.

Materials and Methods

Epitope mapping

As the first step in identifying the epitope recognized by MAb-5H4, cDNA fragments for pZP4 were ligated in an expression vector *pMAL*-c2 (New England BioLab, Beverly, MA). The fragments were excised from the *pUC* 119 constitution containing cDNA encoding the sequence of whole pZP4 polypeptide as described by Hasegawa *et al.* (1994). *pMAL*-pZP4 I was constituted with the 428 bp *Sac*I-*Hind*III fragment that contained cDNA coding for whole pZP4. *pMAL*-pZP4 II was constituted with the 92 bp *Sac*I-*Eco*RI fragment coding for the position of amino acid 1–27 from the amino-terminal region of pZP4. *pMAL*-pZP4 III was constituted with the 346 bp *Sac*I-*Bam*HI fragment coding for amino acid 1–112 from the amino-terminal amino acid of pZP4 (Fig. 1). Excised cDNA fragments included the multicloning region of the original *pUC* 119; thus their size was larger than that expected from the number of amino acids coding for pZP4 peptides. Cloning and expression were carried out in *E. coli* (JM 109). The vector contained *Tac* promoter and *MalE* gene of maltose binding protein. The inserted products of the cDNAs were expressed as hybrid proteins with maltose binding protein by induction with isopropyl-dithiogalactopyranoside (Takara Biochem, Kyoto).

SDS-PAGE and western blotting

Pellets of bacteria induced to express the hybrid recombinant protein were dissolved in sample buffer containing 2% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerine and 0.01% (w/v) bromophenol blue in 50 mmol Tris-HCl l^{-1} , pH 6.8, for SDS-PAGE. After boiling for 5 min, SDS-PAGE was carried out according to Laemmli (1970). The proteins separated on the gel were transblotted onto a polyvinylidene disulfide membrane (Millipore, Bedford, MA) in 10 mmol 3-cyclohexylaminopropane sulfonic acid blotting buffer l^{-1} , pH 11.0 (Matsudaira, 1987). The membrane was blocked for 30 min with 3% (w/v) BSA in 50 mmol Tris-HCl l^{-1} , pH 7.2, containing 200 mmol NaCl l^{-1} (TBS), and followed by incubation with undiluted MAb-5H4 supernatant for 1 h at room temperature as a first reaction. After washing three times with TBS, the membrane was treated with peroxidase-labelled goat anti-mouse IgG (Bio-Rad, Rockville Center, NY) (1:1000) for 30 min at room temperature. After three further washes of the membrane with TBS, the colour development was carried out using 0.3 g 4-chloro-1-naphthol l^{-1} and 0.02% H_2O_2 in TBS.

Fig. 1. Epitope mapping by cDNA of the peptide sequence of zona pellucida glycoprotein ZP4 recognized by the monoclonal antibody MAb-5H4. (a) Schematic diagram of cDNA encoding pig ZP4 and the fragments used for epitope mapping. (b) Agarose gel electrophoresis of DNA fragments excised from pUC119-ZP4. The 428 bp (a), 92 bp (b) and 346 bp (c) bands represent *Sac*I-*Eco*RI, *Sac*I-*Bam*HI and *Sac*I-*Hind*III fragments, respectively. Lane M contained size standards (Marker4; Nippon gene). These DNA fragments were used in constructing the expression vectors for the epitope mapping.

Analysis of antigen determinants

Computer analysis (Karplus and Schulz, 1985) to identify regions of high chain flexibility of proteins was applied to the amino acid sequence of pZP4 as reported by Hasegawa *et al.* (1994). Highly flexible regions are expected to be possible antigen determinants.

Direct binding

Three synthetic peptides were custom prepared by a solid-phase method (Peptide Institute, Osaka). The synthetic peptides were serially diluted from 100 μ mol l^{-1} to 0.01 μ mol l^{-1} in PBS, pH 7.2. One microlitre aliquots containing 100 pmol to 0.01 pmol of diluted samples were dotted on a nitrocellulose membrane (Millipore) and left to dry naturally. After blocking with TBS containing 3% (w/v) BSA, the membrane was incubated in MAb-5H4 supernatant (at 10 mg IgG l^{-1}) for 1 h at room temperature. After washing with TBS for 30 min, the membrane was incubated with peroxidase-labelled goat anti-mouse IgG (Bio-Rad) (1:1000) for 30 min at room temperature. Colour development was carried out as in the western blotting procedure described above.

Inhibition of competitive binding

pZP4 was isolated from pig zona pellucida as described by Koyama *et al.* (1991). Polystyrene 96-well microtitre plates (Falcon, Oxford, CA) were coated with 400 ng of pZP4 per well in 50 mmol sodium carbonate buffer l^{-1} , pH 9.6 overnight at 4°C. After washing three times with PBS, the plates were blocked with PBS containing 1% (w/v) BSA for 30 min at room temperature. The plates were washed three times with PBS and then incubated with a mixture of MAb-5H4 (at 10 mg l^{-1}) and competitors for 2 h at room temperature. The competitors included pZP4 and three synthetic peptides containing amino acid sequences 50–67, 60–79, 70–100 of pZP4. After three washes with PBS, the plates were incubated with peroxidase-labelled goat anti-mouse IgG (Bio-Rad) at a dilution of 1:1000 for 1 h at room temperature. After washing, the colour reaction was developed with 0.2 g *o*-phenylenediamine l^{-1} in 150 mmol citrate-phosphate buffer l^{-1} , pH 5.0 and 0.01% (v/v) H_2O_2 at room temperature for 5 min. Absorbance values were measured at 492 nm using a microplate reader (Bio-Rad).

Immunization

The synthetic 18mer peptide including the 5H4 epitope was conjugated with keyhole limpet haemocyanin (KLH) by the maleimide method (Lerner *et al.*, 1981). ICR mice were injected s.c. with the conjugated protein (20 μ g) in complete Freund's adjuvant (Difco, Detroit, MI). After 2 weeks, the animals were injected with the same immunogen in incomplete Freund's adjuvant followed by three additional booster injections without adjuvant at intervals of 2 days at 2 weeks after the second injection. Blood samples were collected one week after the last booster injection. Antibody production in the sera was assessed by ELISA to the 18mer synthetic peptide or native pZP4 isolated from porcine zona pellucida.

Assessment of antibody production by ELISA

Antibody production against the 50–67 synthetic peptide was assessed by ELISA with native pZP4 or the synthetic peptide used for immunization. A microplate (Falcon) was coated overnight with pZP4 solution at 4°C with 2 μ g protein in each well. After blocking the plate with 1% BSA-PBS, diluted antiserum (1:100 to 1:12 800) was added to the plate, followed by incubation for 1 h at room temperature. After three washings, peroxidase-labelled goat anti-mouse IgG (diluted 1:1000) was used for the second reaction. Colour development was carried out using 0.2 g *o*-phenylenediamine l^{-1} and 0.01% (v/v) H_2O_2 in 150 mmol citrate phosphate buffer l^{-1} , pH 5.0.

In vitro fertilization of pig oocytes

Cumulus-enclosed pig oocytes were collected from ovarian follicles and incubated in TC199 (Flow Laboratories, Costa Mesa, CA) containing 10% (v/v) fetal calf serum (FCS), 1 g sodium pyruvate l^{-1} , 10 iu pregnant mares' gonadotrophin ml^{-1} and 10 iu hCG ml^{-1} in 5% CO_2 in air. After incubation

for 48 h, cumulus masses were treated with 1 g hyaluronidase l^{-1} (Sigma, St Louis, MO) in 0.1% (w/v) polyvinylalcohol in PBS (PVA-PBS) to remove dispersed cumulus cells. The denuded oocytes were added to the fertilization medium containing 10% (v/v) test serum, and incubated for 1 h before the addition of boar spermatozoa. The fertilization medium was TC199 containing 0.1 g sodium pyruvate l^{-1} , 0.55 g glucose l^{-1} , 0.9 g calcium lactate l^{-1} , 0.4 g caffeine l^{-1} , and 10% (v/v) FCS. Boar spermatozoa were prepared from fresh ejaculates to a concentration of $1 \times 10^7 ml^{-1}$ by washing and centrifugation (800 g for 5 min) with PVA-PBS, and then added to the fertilization medium containing the oocytes to a final concentration of 1×10^8 motile spermatozoa l^{-1} . After 18 h, oocytes were washed by gentle pipetting and fixed in acetic acid: ethanol (1:3 v/v) on slides for 3 days at room temperature. Fertilization was assessed by visualization of male pronuclei or enlarged sperm heads in the egg cytoplasm.

Statistical analyses

Statistical analyses were performed using the χ^2 test.

Results

Epitope mapping of MAb-5H4

The sizes of DNA fragments inserted into expression vectors were confirmed by an agarose gel (Fig. 1). The first fragment excised by *SacI/HindIII* of 428 bp corresponds to the cDNA coding for amino acids 1–133 of the pZP4 polypeptide. The second fragment (*SacI/EcoRI*) of 92 bp is a region coding for the 1–27 amino acid sequence of pZP4. The third fragment (*SacI/BamHI*) of 346 bp is a region coding for the 1–112 amino acid sequence. Three recombinant hybrid proteins produced in the transformed bacteria were examined by their reactivities with MAb-5H4 on immunoblotting (Fig. 2). Two transformants, *pMAL-pZP4 I* including the cDNA region for amino acids 1–133 and *pMAL-pZP4 III* including the cDNA region for amino acids 1–112, showed positive reactions, while *pMAL-pZP4 II* including the cDNA region for 1–27 did not. This result suggested that the MAb-5H4 epitope is present in the amino acid positions 28–112 in pZP4.

Determination of MAb-5H4 epitope sequence

Putative antigenic determinants of pZP4 were predicted (Fig. 3). Three highly flexible regions were shown in amino acids 28–112. On the basis of this result, three peptides corresponding to amino acid sequences 50–67, 60–79 and 70–100 were synthesized. The dot immunoassay clearly revealed that only one synthetic peptide (the 50–67 amino acid sequence) reacted with MAb-5H4 (Fig. 4). Since the 60–67 amino acid sequence is contained in both the reactive 50–67 peptide and in the non-reactive 60–79 peptide, the MAb-5H4 epitope is therefore present on the 50–59 amino acid sequence which corresponds to CTYVLDPENL.

The specific reactivity of the synthetic peptide for MAb-5H4 was confirmed by competitive-binding inhibition against immobilized native pZP4. A dose-dependent inhibition of the

Fig. 2. Immunological detection of MAb-5H4 epitope of pig zona pellucida glycoprotein ZP4 produced by transformants. *Escherichia coli* lysate was separated by SDS-PAGE, blotted to PVDF membrane, and probed with MAb-5H4. The electrophoresed samples were bacterial lysate transformed by an expression vector pMAL containing *SacI*-*EcoRI* (a), *SacI*-*BamHI* (b) and *SacI*-*HindIII* (c) fragments. The apparent molecular masses were determined using low-range prestained markers indicated to the left of lane a.

Fig. 3. Flexibility assay of pig zona pellucida glycoprotein ZP4. This assay was carried out according to the program of Karplus and Schulz (1985). Five highly flexible regions were shown in ZP4 polypeptide. Three synthetic peptides (positions 50–67, 60–79, 70–100) were custom-prepared for determination of the epitope of the MAb-5H4. The determined epitope sequence was coincident with the second flexible region shown shadowed.

native pZP4 binding to MAb-5H4 by the 50–67 amino acid synthetic peptide, but not the other two synthetic peptides, was observed (Fig. 5). The degree of inhibition with the synthetic peptide was the same as that for native pZP4 at each concentration.

Fig. 4. Dot immunoassay of three synthetic peptides using MAb-5H4. Three synthetic peptides corresponding to amino acid positions 50–67, 60–79 and 70–110 of pig zona pellucida glycoprotein ZP4 were custom-prepared based on the peptide mapping and computer analysis. For analysis, 1 μ l of the peptides sample was dotted on nitrocellulose membrane at concentrations of 100 pmol to 0.01 pmol μ l⁻¹ in serial dilutions. The membrane was blocked with 3% BSA containing TBS and followed by immunological detection with MAb-5H4. Only one synthetic peptide, 50–67, showed a positive reaction at the concentration of 1 pmol μ l⁻¹.

Fig. 5. Competitive binding inhibition of MAb-5H4 binding to native pig zona pellucida glycoprotein pZP4 by synthetic peptides corresponding to amino acid sequences 50–67, 60–79 and 70–110. Microtitre plates coated with native pZP4 were reacted with the MAb-5H4 in the presence of synthetic peptides or native pZP4 at the indicated concentrations. Note that the MAb-5H4 binding to immobilized native pZP4 was inhibited in a dose-dependent fashion by the synthetic peptide 50–67 (▲) but not the other two synthetic peptides 60–79 (□) and 70–110 (●). The degree of inhibition by the peptide 50–67 (▲) was almost the same as that of native pZP4 (○).

Species distribution of MAb-5H4 epitope

MAb-5H4 reacted with intact zona pellucida of pigs, humans and rabbits but not mice (Fig. 6). The amino acid sequences from the four species corresponding to the MAb-5H4 epitope sequence are compared (Fig. 7). Two positions, 53 (valine) and 58 (asparagine) are replaced with isoleucine (53) and lysine (58) in rabbits and humans. Four positions, 53, 56, 58 and 59 are replaced in mice. The positions 56 (proline) and 59 (leucine) seem to be critical for the MAb-5H4 epitope. The replacement positions 53 and 58 did not influence the MAb-5H4 epitope.

Fig. 6. Immunofluorescent staining of oocytes from (a) pigs, (b) humans, (c) rabbits and (d) mice with the MAb-5H4. Pig and human oocytes were collected from ovarian follicles. In rabbits and mice, ovulated oocytes were obtained from the oviducts. The collected oocytes were treated with undiluted supernatant of the MAb-5H4. After washing, they were treated with FITC-labelled goat anti-mouse IgG (1:200). All species except mice showed positive reactions.

	50	51	52	53	54	55	56	57	58	59
Pig ZP4(ZP1)	C	T	Y	V	L	D	P	E	N	L
Human ZP2	.	.	.		.	.	.	.	K	.
Rabbit R75K	.	.	.		.	.	.	.	K	.
Mouse ZP2	.	.	.	A	.	.	L	.	R	F

Fig. 7. Comparison of MAb-5H4 epitope sequence in pigs (positions 50–59 of zona pellucida glycoprotein ZP4) with corresponding sequences in three other species. The amino acid sequences of human ZP2, rabbit 75K, mouse mZP2 were according to Liang and Dean (1993b); Lee *et al.* (1993) and Liang *et al.* (1990), respectively.

Immunogenicity of the synthetic peptide (50–67)

The 18mer synthetic peptide 50–67 induced antibody production in mice when conjugated to keyhole limpet haemocyanin (KLH) (Fig. 8). The antisera reacted with the synthetic peptide 50–67 and also with native pZP4. The antibody titres to pZP4 of the anti-synthetic peptide antiserum showed 1:3200–1:6400 dilution. This value is equivalent to the titres of the antiserum raised to the native pZP4 glycoprotein. The mouse antiserum also reacted with intact pig zona pellucida by indirect immunofluorescent staining of porcine oocytes but not of other species (Fig. 9).

Blocking of fertilization

When the mouse antiserum to the synthetic peptide 50–67 was added to the fertilization medium, numerous spermatozoa attached to the zona pellucida immediately after the insemination. However, only a few spermatozoa remained on the zona pellucida after 24 h and none had penetrated into the zona pellucida.

The fertilization rate of pig oocytes treated with mouse antiserum to the synthetic peptide 50–67 was significantly reduced compared with that for the control group (Table 1).

Discussion

In the study reported here, the amino acid sequence of the epitope recognized by MAb-5H4, which possessed the ability to block fertilization, was determined and the antibody produced to the epitope sequence was characterized. Previously,

Fig. 8. Assessment of mouse antibody production to the synthetic peptide corresponding to the amino acid sequence 50–67 of pig zona pellucida glycoprotein ZP4 by ELISA. The synthetic peptide 50–67 conjugated with keyhole limpet haemocyanin (KLH) was used for immunization in mice. Antiserum against KLH-conjugated peptide 50–67 (●) reacted with the synthetic peptide 50–67, while the antiserum from the control animal immunized with KLH (▲) did not. The antiserum produced reacted with (a) the peptide 50–67 and also with (b) native pZP4 (○).

Fig. 9. Immunofluorescent staining of oocytes with the mouse antiserum to the synthetic peptide corresponding to the amino acid sequence 50–67 of pig zona pellucida glycoprotein ZP4. The collected oocytes were treated with the antiserum to keyhole limpet haemocyanin (KLH) as control (a) or KLH-conjugated peptide 50–67 (b, c, d, e) at a dilution of 1:500. Oocytes were obtained from (a, b) pigs, (c) humans, (d) rabbits and (e) mice.

we had cloned the cDNA encoding a pig zona pellucida glycoprotein, pZP1, and found that the mature glycoproteins of pZP4 and pZP2 were derived from the parental molecule of the pZP1 glycoprotein (Hasegawa *et al.*, 1994). pZP4 constitutes the amino-terminal region (positions 1–133) of pZP1, while pZP2 constitutes the carboxy-terminal region of pZP1 (positions 134–680). The MAb-5H4 was found to recognize pZP1 as well as pZP4 (Koyama *et al.*, 1991). In the work reported here the peptide sequence recognized by

Table 1. Effect of mouse antiserum to the synthetic peptide epitope corresponding to amino acid positions 50–67 of zona pellucida glycoprotein ZP4 on the fertilization of pig oocytes *in vitro*

Mouse antiserum	Number of eggs used	Number of eggs fertilized	Fertilization rate (%)
Anti-KLH ^a	49	37	75.5
Anti-KLH-peptide 50–67	46	11	23.9 ^b

^aKeyhole limpet haemocyanin.

^b $P < 0.001$ (χ^2 test).

MAB-5H4 epitope and the immunogenicity of the synthetic 18mer peptide containing the epitope sequence are described.

Epitope mapping was performed using cDNA fragments coding for several different regions of the pZP4 polypeptide. The epitope mapping revealed that the region 28–112 contained the epitope of MAB-5H4. Flexibility analysis of the pZP4 polypeptide showed three putative antigenic determinants in this region. Three peptides, overlapping the amino- and carboxy-terminal ends, were custom-prepared based on the epitope mapping and the flexibility analysis. The MAB-5H4 reacted with only one of the three synthetic peptides and the epitope was determined to be a sequence of 10 amino acids CTYVLDPENL. The specificity of the synthetic peptide was confirmed for MAB-5H4 by competitive binding inhibition ELISA against native pZP4. The dose–response binding inhibition by the synthetic peptide 50–67 exhibited a similar binding inhibition curve to that of native pZP4, suggesting that the antigen to the synthetic peptide 50–67 possesses a similar affinity to native pZP4 as for MAB-5H4. This suggests that the MAB-5H4 recognizes a sequential peptide epitope but not a steric- or carbohydrate-contributed conformational structure.

A comparison of the amino acid sequences of the corresponding region from four different species (pig and human (Liang and Dean, 1993b), rabbit (Lee *et al.*, 1993), and mouse (Liang *et al.*, 1990)) revealed that position 56 (proline) and position 59 (leucine) are essential for the epitope structure of MAB-5H4; MAB-5H4 recognizes zona pellucida from pigs, humans and rabbits but not from mice. This may be related to the capacity of proline to create a fixed kink in a polypeptide chain. However, changing position 53 from valine to alanine and position 58 from asparagine to arginine may also be critical. Changing position 53 from valine to isoleucine and position 58 from asparagine to lysine do not appear to be so important for the epitope structure.

Since the epitope seems to be an attractive target antigen for developing a contraceptive vaccine, the immunogenicity of the synthetic peptide 50–67 containing the epitope was examined. The peptide when conjugated with KLH induced antibody production in mice. The mouse antiserum reacted not only with the synthetic peptide 50–67 but also with the native pZP4 glycoprotein and intact pig zona pellucida. These results suggest that the epitope is conserved in the 18mer synthetic peptide 50–67 and is exposed on the outside of the molecule sufficiently to be recognized by immunocompetent cells. This characteristic property of the epitope is important for blocking

fertilization by the corresponding antibody. In fact, the mouse antiserum to the synthetic peptide 50–67 markedly inhibited *in vitro* fertilization of pig oocytes.

The fertilization rate of pig oocytes treated with mouse antiserum to the synthetic peptide 50–67 was significantly reduced compared with control serum. This low fertilization rate is thought to be due to the antiserum blocking the second step of sperm binding to the zona pellucida. In mice, mZP2 was proposed as a secondary sperm receptor that retains acrosome-reacted spermatozoa on the zona pellucida and accelerates sperm penetration (Bleil *et al.*, 1988). It has been suggested that pZP1 is the counterpart of mouse ZP2 owing to the similarity of amino acid sequences and the results of enzymatic digestion of these two glycoproteins (Florman *et al.*, 1984; Hasegawa *et al.*, 1994; Taya *et al.*, 1995). The present observations that the antiserum to the synthetic peptide 50–67 inhibits fertilization but not the initial binding of spermatozoa to zona pellucida suggests that pZP1 is functioning as a secondary sperm receptor in pigs.

Fertilization could also be inhibited by other mechanisms, such as steric hindrance of zona conformation and changes in the susceptibility to sperm fertilization enzymes. Therefore, there are various sites on the zona pellucida where the antibodies could inhibit fertilization.

When native zona antigens from heterospecies are used for immunization, the immunized animals often produce self-reactive anti-zona antibodies which result in infertility. However, most of the immunized animals also suffer from ovarian failure (Sacco *et al.*, 1983; Skinner *et al.*, 1984; Mahi-Brown *et al.*, 1985, 1988; Hasegawa *et al.*, 1988; Henderson *et al.*, 1988; Keenan *et al.*, 1991). Paterson *et al.* (1992) observed a marked reduction of primordial follicles and hormonal disturbances in marmosets that were immunized with highly purified zona antigens including deglycosylated preparations. These undesirable side effects are thought to be elicited by T-cell-dependent cytotoxicity.

In contrast, the antigens used for immunization from homologous zona pellucida induced only low titres of antibodies, insufficient to induce infertility in the immunized animals. These low titres probably result from immunological tolerance to self antigens (Gwatkin *et al.*, 1977; Sacco, 1979; Wood *et al.*, 1981). In general, it is difficult to induce self-reactive autoantibodies that can inhibit fertilization without inducing T-cell-dependent cytotoxicity with accompanying ovarian failure. However, the fact that anti-zona pellucida autoantibodies could be obtained by heteroimmunization indicates that B-cell clones reactive to self-zona-pellucida antigens are not eliminated. Thus, it should prove possible to construct a vaccine that could produce the self-reactive antibody by using interspecies crossreactive zona antigens. For this purpose, site-specific peptide analysis and antigen modification by protein engineering or recombinant protein technology would be necessary. It has been shown that the 16mer synthetic peptide of murine mZP3 produced autoantibodies to zona pellucida and induced long-term infertility in female mice (Millar *et al.*, 1989). Later, it was found that the 16mer peptide contained not only the B-cell epitope but also the T-cell epitope which induced oophoritis in some strains (Lou and Tung, 1993). This demonstrated that it is possible that the contraceptive effect of the B-cell epitope can be segregated from undesired side-effects by the T-cell epitope.

Recently, it has become possible to investigate the target antigens of the zona pellucida at epitope levels, since full-length cDNAs have been reported in various mammals including mice (Ringuette *et al.*, 1986; Liang *et al.*, 1990), hamsters (Kinlock *et al.*, 1990), rabbits (Schwoebel *et al.*, 1991; Lee *et al.*, 1993), humans (Chamberlin and Dean, 1990; Liang and Dean, 1993b), marmosets (Thillai-Koothan *et al.*, 1993), pigs (Harris *et al.*, 1994; Taya *et al.*, 1995), dogs, cats and cows (Harris *et al.*, 1994). On the basis of amino acid sequences deduced from cDNA, the immunogenicity of the synthetic peptides and the biological effects of resultant antisera raised have been examined. Yurewicz *et al.* (1993b) reported that a site-directed antibody produced against a synthetic 11mer peptide corresponding to the amino-terminal region of pZP3a partially interfered with sperm binding of boar spermatozoa to porcine zona pellucida. Gupta *et al.* (1994) also reported that the synthetic peptide positions 323–341 of human ZP3 induced antibody production even though they did not show any biological effects of the antiserum. However, it is difficult to find promising antigens for vaccine development using peptide synthesis because numerous peptides must be synthesized for screening of the appropriate antigens. In this regard, the strategy using monoclonal antibodies is useful for detection of effective antigen epitopes in high molecular mass proteins with numerous complicated epitopes. Many researchers have produced monoclonal antibodies against porcine zona pellucida glycoproteins and characterized the antigens recognized by them (Drell and Dunbar, 1984; Isojima *et al.*, 1984; Timmons *et al.*, 1987; Koyama *et al.*, 1991; Bagavant *et al.*, 1993; Gupta *et al.*, 1993). However, few epitope structures corresponding to the established monoclonal antibodies have been determined. Millar *et al.* (1989) reported that a 16mer synthetic peptide of murine mZP3 recognized by a fertilization blocking monoclonal antibody could induce infertility in mice. However, according to their report, the epitope was species-specific to mice and, therefore, their data were of limited interest to workers interested in the development of human contraceptive vaccine. By contrast, the MAb-5H4 has been shown to react with zona pellucida of humans, dogs, cats, cows and rabbits as well as pigs, and it is possible to conduct a model experiment for human studies in several different species using their epitope.

The authors thank Y. Hirao and K. Kano for their technical advice in the *in vitro* fertilization experiments. This research was supported by Grant-in-Aid of Scientific Research (No. 06454481) from the Ministry of Education, Science and Culture, and by Science Research Promotion Funds from the Japan Private School Promotion Foundation Tokyo, Japan.

References

- Aitken RJ, Paterson M and Thillai-Koothan P (1993) Contraceptive vaccine *British Medical Bulletin* **49** 88–99
- Bagavant H, Yurewicz EC, Sacco AG, Talwer GP and Gupta SK (1993) Delineation of epitopes on porcine zona pellucida relevant for binding of sperm to oocytes using monoclonal antibodies *Journal of Reproductive Immunology* **23** 265–279
- Bleil JD, Greve JM and Wassarman PM (1988) Identification of a second sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs *Developmental Biology* **128** 376–385
- Chamberlin EM and Dean J (1990) Human homology of the mouse sperm receptor *Proceedings of the National Academy of Sciences USA* **87** 6014–6018
- Drell DW and Dunbar BS (1984) Monoclonal antibodies to rabbit and pig zonae pellucidae distinguish species-specific and shared antigenic determinants *Biology of Reproduction* **30** 445–457
- Florman HM, Bechtol KB and Wassarman PM (1984) Enzymatic dissection of the functions of the mouse egg's receptor for sperm *Developmental Biology* **106** 243–255
- Gupta SK, Bagavant H, Chadha K, Gupta M, Yurewicz EC and Sacco AG (1993) Mapping of immunogenic domains on porcine zona pellucida 3 alpha and beta glycoproteins by murine monoclonal antibodies *American Journal of Reproductive Immunology* **30** 95–100
- Gupta SK, Kaul R, Rajalakshmi S, Sahai P, Yurewicz EC and Sacco AG (1994) Immunoreactivity with native zona pellucida of antibodies against a 19 amino acid synthetic peptide corresponding to human ZP3 *Journal of Reproductive Immunology* **27** 241–247
- Gwatkin RBL, Williams DT and Carlo DJ (1977) Immunization of mice with heat-solubilized hamster zonae: production of anti-zona antibody and inhibition of fertility *Fertility and Sterility* **28** 871–877
- Harris JD, Hibler DW, Fontenot GK, Hsu KT, Yurewicz EC and Sacco AG (1994) Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families *DNA Sequence* **4** 361–393
- Hasegawa A, Koyama K and Isojima S (1988) Immunogenicity of a 92 kDa component of porcine zona pellucida isolated using a monoclonal antibody (3A4-2G1) exclusively cross-reactive with porcine and human zonae pellucidae *Journal of Reproductive Immunology* **14** 73–82
- Hasegawa A, Koyama K, Inoue M, Takemura T and Isojima S (1992) Antifertility effect of active immunization with ZP4 glycoprotein family of porcine zona pellucida in hamsters *Journal of Reproductive Immunology* **22** 197–210
- Hasegawa A, Koyama K, Okazaki Y, Sugimoto M and Isojima S (1994) Amino acid sequence of a porcine zona pellucida glycoprotein ZP4 determined by peptide mapping and cDNA cloning *Journal of Reproduction and Fertility* **100** 245–255
- Hedrick JL (1993) The pig zona pellucida: sperm binding ligands, antigens and sequence homologies. In *Reproductive Immunology* Vol. 97 pp 59–65 Eds F Dondero and PM Johnson. Raven Press, New York
- Henderson CJ, Hulme MJ and Aitken RJ (1988) Contraceptive potential of antibodies to the zona pellucida *Journal of Reproduction and Fertility* **83** 325–343
- Isahakia MA and Bambara CS (1992) Antisperm and anti-ovum vaccines. The selection of candidate antigens and the outcome of preclinical studies *Scandinavian Journal of Immunology* **36** (Supplement 11) 118–122
- Isojima S, Koyama K, Hasegawa A, Tsunoda Y and Hanada A (1984) Monoclonal antibodies to porcine zona pellucida antigens and their inhibitory effects on fertilization *Journal of Reproductive Immunology* **6** 77–87
- Karplus PA and Schulz GE (1985) Prediction of chain flexibility in proteins: a tool for the selection of peptide antigens *Naturwissenschaften* **72** 212–213
- Keenan JA, Sacco AG, Subramanian MG, Kruger M, Yurewicz EC and Moghissi KS (1991) Endocrine response in rabbits immunized with native, versus de-glycosylated, porcine zona pellucida antigens *Biology of Reproduction* **44** 150–156
- Kinlock R, Ruiz-Seiler B and Wassarman PM (1990) Genomic organization and polypeptide primary structure of zona pellucida glycoprotein hZP3: the hamster sperm receptor *Developmental Biology* **142** 414–421
- Kinlock R, Sakai Y and Wassarman PM (1995) Mapping the mouse ZP3 combining site for sperm by exon swapping and site-directed mutagenesis *Proceedings of the National Academy of Sciences USA* **92** 263–267
- Koyama K, Hasegawa A, Inoue M and Isojima S (1991) Blocking of human sperm–zona interaction by monoclonal antibodies to a glycoprotein family (ZP4) of porcine zona pellucida *Biology of Reproduction* **45** 727–735
- Laemmli UK (1970) Cleavage of structure proteins during the assembly of the head of bacteriophage T4 *Nature* **227** 680–685
- Lee VH, Schwoebel E, Parasad S, Cheung P, Timmons TM, Cook R and Dunbar BS (1993) Identification and structural characterization of the 75-kDa rabbit zona pellucida protein *Journal of Biological Chemistry* **268** 12412–12417
- Lerner RA, Green N, Alexander H, Liu F, Sutcliffe JG and Shinnick TM (1981) Chemically synthesized peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles *Proceedings of the National Academy of Sciences USA* **78** 3403–3407

- Liang L and Dean J (1993a) Oocyte development: molecular biology of zona pellucida *Vitamins and Hormones* **47** 115–159
- Liang L and Dean J (1993b) Conservation of mammalian secondary sperm receptor genes enables the promoter of the human gene to function in mouse oocytes *Developmental Biology* **156** 399–408
- Liang L, Chamow SM and Dean J (1990) Oocyte-specific expression of mouse ZP-2: developmental regulation of the zona pellucida genes *Molecular and Cellular Biology* **10** 1507–1515
- Lou Y and Tung KS (1993) T cell peptide of a self-protein elicits autoantibody to the protein antigen. Implications for specificity and pathogenetic role of antibody in autoimmunity *Journal of Immunology* **151** 5790–5799
- Mahi-Brown CA, Yanagimachi R, Hoffman JC and Huang TFH, Jr (1985) Fertility control in the bitch by active immunization with porcine zonae pellucidae: use of different adjuvants and patterns of estradiol and progesterone levels in estrus cycles *Biology of Reproduction* **32** 761–772
- Mahi-Brown CA, Yanagimachi R, Nelson ML, Yanagimachi H and Palumbo N (1988) Ovarian histopathology of bitches immunized with porcine zonae pellucidae *American Journal of Reproductive Immunology* **18** 94–103
- Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes *Journal of Biological Chemistry* **262** 10 035–10 038
- Millar SE, Chamow SM, Baur AW, Oliver C, Robey F and Dean J (1989) Vaccination with a synthetic zona pellucida peptide produces long-term contraception in female mice *Science* **246** 935–938
- Naz RK, Sacco A, Singh O, Pal R and Talwer G (1995) Development of contraceptive vaccines for humans using antigens derived from gametes (spermatozoa and zona pellucida) and hormones (human chorionic gonadotrophin): current status *Human Reproduction Update* **1** 1–118
- Oikawa T and Yanagimachi R (1975) Block of hamster fertility by anti-ovary antibody *Journal of Reproduction and Fertility* **45** 487–494
- Paterson M, Koothan PT, Morris KD, O'Byrne KT, Braude P, Williams A and Aitken RJ (1992) Analysis of the contraceptive potential of antibodies against native and deglycosylated porcine ZP3 *in vivo* and *in vitro* *Biology of Reproduction* **46** 523–534
- Ringette MJ, Chamberlin ME, Baur AW, Solareski DA and Dean J (1986) Molecular analysis of cDNA coding for ZP3, a sperm binding protein of the mouse zona pellucida *Developmental Biology* **127** 287–295
- Sacco AG (1977) Antigenic cross-reactivity between human and pig zona pellucida *Biology of Reproduction* **16** 164–173
- Sacco AG (1979) Inhibition of fertility in mice by passive immunization with antibodies to isolated zonae pellucidae *Journal of Reproduction and Fertility* **56** 533–537
- Sacco AG and Yurewicz EC (1993) Use of porcine zona pellucida 55K macromolecules as target antigens for contraceptive vaccine development *Reproductive Immunology* **97** 75–84
- Sacco AG, Subramanian MG, Yurewicz EM, DeMayo J and Dukelow WR (1983) Heteroimmunization of squirrel monkeys (*Saimiri sciureus*) with a purified porcine zona pellucida antigen (PPZA): immune response and biologic activity of antiserum *Fertility and Sterility* **39** 350–358
- Sacco AG, Yurewicz EM, Subramanian MG and Matzat PD (1989) Porcine zona pellucida: association of sperm receptor activity with the glycoprotein component of the $M_r = 55,000$ family *Biology of Reproduction* **41** 523–532
- Schwoebel ED, Prasad S, Timmons TM, Cook R, Kimura H, Dunbar B, Cheung P, Skinner S, Avery SE, Wilkins B and Dunbar BS (1991) Isolation and characterization of a full-length cDNA encoding the 55-kDa rabbit zona pellucida protein *Journal of Biological Chemistry* **266** 7214–7219
- Skinner SM, Mills T, Kirchick HJ and Dunbar BS (1984) Immunization with zona proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin induced steroid secretion *Endocrinology* **115** 2418–2432
- Taya T, Yamasaki N, Tsubamoto H, Hasegawa A and Koyama K (1995) Cloning of a cDNA coding for porcine zona pellucida glycoprotein ZP1 and its genomic organization *Biochemical and Biophysical Research Communications* **207** 790–799
- Thillai-Koothan P, van Duin M and Aitken RJ (1993) Cloning, sequencing and oocyte-specific expression of the marmoset sperm receptor protein, ZP3 *Zygote* **2** 1–9
- Timmons TG, Maresh GA, Bundman DS and Dunbar BS (1987) Use of specific monoclonal antibodies to define distinct antigens of the porcine zonae pellucidae *Biology of Reproduction* **36** 1275–1287
- Tsunoda Y and Chang MC (1978) Effects of antisera against eggs and zonae pellucidae on fertilization and development of mouse eggs *in vivo* and *in culture*. *Journal of Reproduction and Fertility* **54** 233–237
- Wassarman PM (1990) Profile of a mammalian sperm receptor *Development* **108** 1–17
- Wassarman PM and Mortillo S (1991) Structure of the mouse egg extracellular coat, the zona pellucida *International Review of Cytology* **130** 85–110
- Wood DM, Liu CL and Dunbar BS (1981) Effect of alloimmunization and heteroimmunization with zona pellucida on fertility in rabbits *Biology of Reproduction* **23** 439–450
- Yurewicz EC, Pack BA, Armant DR and Sacco AG (1993a) Porcine zona pellucida ZP3 α glycoprotein mediates binding of the biotin-labeled M_r 55,000 family (ZP3) to boar sperm membrane vesicles *Molecular Reproduction and Development* **36** 382–389
- Yurewicz EC, Zhang S and Sacco AG (1993b) 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 *Journal of Reproduction and Fertility* **98** 147–152