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. Koyama¹* and S. Isojima²

¹Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan; and ²Advanced Fertility Center, Fuchu Hospital, Izumi-fuchu, 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 CTYVLPENL, 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, pZP3α and pZP3β. pZP1 is divided into pZP2 and pZP4 by reduction of S-S bonds (Hedrick, 1993; Hasegawa et al., 1994). pZP3α 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 (Ishakia 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 depletion 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 1993).
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 CTYVD-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 region as described by Hasegawa et al. (1994). pMAL-pZP4 I was constituted with the 428 bp SacI-HindIII fragment that contained cDNA coding for whole pZP4. pMAL-pZP4 II was constituted with the 92 bp SacI-EcoRI 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 SacI-BamHI 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 1−1, 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 difluoride membrane (Millipore, Bedford, MA) in 10 mmol 3-cyclohexylaminopropanesulfonic acid blotting buffer 1−1, pH 11.0 (Matsuda, 1987). The membrane was blocked for 30 min with 3% (w/v) BSA in 50 mmol Tris–HCl 1−1, pH 7.2, containing 200 mmol NaCl 1−1 (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 further washes of the membrane with TBS, the colour development was carried out using 0.3 g 4-chloro-1-naphthol 1−1 and 0.02% H2O2 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 SacI-EcoRI, SacI-BamHI and SacI-HindIII 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 pmol 1−1 to 0.01 pmol 1−1 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 1−1) 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 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 1 M, 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 (10 mg IgG/1 M) 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 1 M in 150 mmol citrate-phosphate buffer 1 M, pH 5.0 and 0.1% (v/v) H2O2 at room temperature for 5 min. Absorbance values were measured at 492 nm using a microplate reader (Bio-Rad).

Immunization

The synthetic 18 mer peptide including the SH4 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 µg) 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 18 mer 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 µg 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 1 M and 0.01% (v/v) H2O2 in 150 mmol citrate phosphate buffer 1 M, 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 1 M, 10 µg pregnant mares' gonadotrophin ml 1 M and 10 µl hCG ml 1 M in 5% CO2 in air. After incubation for 48 h, cumulus masses were treated with 1 g hyaluronidase 1 M (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 1 M, 0.55 g glucose 1 M, 0.9 g calcium lactate 1 M, 0.4 g caffeine 1 M, and 10% (v/v) FCS. Boar spermatozoa were prepared from fresh ejaculates to a concentration of 1 x 106 ml 1 M 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 x 104 motile spermatozoa 1 M. 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 χ2 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 II 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
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.

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.
Epitope peptide of MAb-5H4 against porcine ZP4

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 · · · I · · · · · K ·
Rabbit R7SK · · · A · · · L · R F
Mouse ZP2 · · · I · · · · · K ·

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.

50-59 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 antisera showed 1:3200–1:6400 dilution. This value is equivalent to the titres of the antisera raised to the native pZP4 glycoprotein. The mouse antisera 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).

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.

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, 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

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 antisera from the control animal immunized with KLH (▲) did not. The antisera 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.
**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

<table>
<thead>
<tr>
<th>Mouse antiserum</th>
<th>Number of eggs used</th>
<th>Number of eggs fertilized</th>
<th>Fertilization rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-KLH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49</td>
<td>37</td>
<td>75.5</td>
</tr>
<tr>
<td>Anti-KLH-peptide 50–67</td>
<td>46</td>
<td>11</td>
<td>23.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Keyhole limpet haemocyanin.
<sup>b</sup>P < 0.001 (χ<sup>2</sup> 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 C1YYLDPENL. 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 spermatozoon 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 spermatozoon 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., 1983, 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 (Gwaltlin 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 (Ringuelet 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 antisera. However, it is difficult to find promising antigens for vaccine development using peptide synthesis because numerous peptides must by 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 10mer 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


Chamberlin EM and Dean J (1990) Human homology of the mouse sperm receptor Proceedings of the National Academy of Sciences USA 87 6014–6018


Harris JD, Hiibler 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


Ishakia MS 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


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
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 Yuwewicz EC (1993) Use of porcine zona pellucida 55K macromolecules as target antigens for contraceptive vaccine development. Reproductive Immunology. 47 75–84
Wassarman PM and Mortillo S (1991) Structure of the mouse egg extracellular coat, the zona pellucida. International Review of Cytology. 130 85–110
Yuwewicz EC, Pack BA, Armant DR and Sacco AG (1993a) Porcine zona pellucida glycoprotein mediates binding of the bioin-labeled M, 55,000 family (ZP3) to boar sperm membrane vesicles. Molecular Reproduction and Development. 36 382–389