Contraceptive responses of mice immunized with purified recombinant mouse zona pellucida subunit 3 (mZP3) proteins

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Mouse zona pellucida subunit 3 (mZP3) was tested for efficacy as an immunocontraceptive antigen by comparing the fertility of mice immunized with recombinant mZP3 proteins. Recombinant protein was expressed using either the vaccinia virus T7 mammalian (vmZP3 protein) or baculovirus insect cell (bmZP3 protein)-expression systems. Female BALB/c or wild mice were immunized by i.p. injection using Freund's complete adjuvant and boosted three times with affinity purified recombinant proteins in Freund's incomplete adjuvant. Most mice developed antibodies that crossreacted to the respective mZP3 antigens by ELISA or western blot. In BALB/c mice

immunized with vmZP3, fertility and mean litter size were reduced transiently to 25% and 10%, respectively, of those of control mice. However, immunization with bmZP3 did not affect either the fertility or mean litter sizes in BALB/c or wild mice immunized with bmZP3. The results demonstrate that reduction in fertility can be achieved in female BALB/c mice immunized using Freund's adjuvants and recombinant mZP3 protein produced in a mammalian, but not an insect, cell-expression system. Arguments are presented for the likely role of glycosylation of the mZP3 antigen in inducing contraceptive immune responses.

Introduction

Contraception by induced immunity (immunocontraception) relies on the identification and delivery of an effective antigen. Active immunization of animals with protein extracts from spermatozoon or egg has been used to identify a number of candidate sperm and egg proteins with immunocontraceptive potential (Frayne and Hall, 1999; Dunbar *et al.*, 2002). Among the most effective have been the complex glycoproteins that form the zona pellucida (ZP) matrix that surrounds and protects the egg. Antigens derived from the ZP protein subunits have shown considerable potential for use in vaccines for fertility control in mammals. Numerous studies have reported that infertility can be induced by direct immunization of animals using whole pig zona pellucida (Dietl *et al.*, 1982; Mahi-Brown *et al.*, 1985; Bhatnagar *et al.*, 1992; Hasegawa *et al.*, 1992; Miller *et al.*, 1999; Li *et al.*, 2002), purified homologous and heterologous ZP proteins or protein fragments (Mahi-Brown, 1996; Paterson *et al.*, 1998; Kerr *et al.*, 1999; Govind and Gupta, 2000; Martinez and Harris, 2000; Lea *et al.*, 2002; Srivastava *et al.*, 2002), and ZP peptides (Millar *et al.*, 1989; Lou *et al.*, 1995; Mahi-Brown, 1996; Sun *et al.*, 1999; Hardy *et al.*, 2002), although the extent and duration of infertility obtained varies considerably among such studies.

The ZP exhibits considerable heterogeneity in both structure and immunogenicity between species due to differences in amino acid sequences and different post-translational modifications that include extensive *N*- and *O*-linked glycosylation and sulphation (Prasad *et al.*, 2000). In mice, the ZP comprises three proteins termed ZP1, ZP2 and ZP3 (Rankin and Dean, 2000). The mouse ZP3 subunit (mZP3) is synthesized as a 424 amino acid precursor molecule with a predicted molecular mass of 46 kDa. It undergoes a series of post-translational modifications within the oocyte, including signal sequence cleavage, glycosylation and furin protease cleavage (Ringuette *et al.*, 1988; Epifano *et al.*, 1995; Litscher *et al.*, 1999) before it is secreted, and ultimately becomes incorporated into the ZP as an 83 kDa glycoprotein (Bleil and Wassarman, 1980a; Shimizu *et al.*, 1983; Beebe *et al.*, 1992; Litscher *et al.*, 1999).

Despite a wealth of data supporting an essential role for glycosylation in fertilization, the importance of such post-translational modifications on the immunogenicity and contraceptive potential of ZP3 is still uncertain. One of the most effective ZP antigens is whole pig zona pellucida and here the contraceptive effect appears to require the induction of strong antibody responses to glycosylated residues on the native zona pellucida proteins (Barber and Fayerer-Hosken, 2000). A few reports have indicated that non-glycosylated bacterially expressed ZP antigens may be effective immunogens in dogs (Srivastava *et al.*, 2002), baboons (Govind and Gupta, 2000) and mice (Zhang *et al.*, 1997), although

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in all these studies numbers of animals used were low, multiple immunizations were required, and only transient or incomplete infertility was reported.

In direct contrast, live recombinant virus vaccines expressing mZP3, based either on ectromelia virus (Jackson *et al.*, 1998) or murine cytomegalovirus (Chambers *et al.*, 1999; Lloyd *et al.*, 2003), induce highly effective and long-lived contraceptive responses in infected mice. Nevertheless, it is not possible to determine from these experiments whether infertility in the infected animals was due to expression of highly antigenic recombinant mZP3 by the viruses, or whether viral replication provided an essential adjuvant effect. Also, there are no reports on whether purified recombinant ZP3 proteins produced in eukaryotic expression systems can induce infertility in mice in the absence of virus replication, nor on whether appropriate glycosylated residues are required to induce robust contraceptive responses.

This report describes the effect of immunizing mice with purified recombinant mZP3 proteins to determine whether the proteins, in the absence of viral replication, are able to induce long-lived immunocontraceptive responses in mice. The study compared the relative immunogenicities and contraceptive effects of immunizing mice with mZP3 produced in two different expression systems (either insect or mammalian), in which different levels and types of post-translational modification could be achieved, to test whether the expression system chosen to produce recombinant protein has an effect on the immunogenicity or contraceptive potential of mZP3.

Materials and Methods

mZP3 peptide

The mZP3 peptide NH₂-CSNSSSSQFIHGPR-COOH representing amino acids 328–342 of mZP3 (GenBank Accession No. M20026) and previously shown to include an immunocontraceptive epitope of mZP3 (Millar *et al.*, 1989) was synthesized at a minimum purity of 95% by Mimotopes Pty Ltd (Clayton, Victoria). This peptide was used only in ELISA assays and was not used for immunizations.

Expression and purification of recombinant mZP3 using baculovirus

The entire mZP3 cDNA (GenBank Accession No. M20026) inserted into pGEM7Z (plasmid pZP3) has been described by Jackson *et al.* (1998). A 1308 bp *Bam*HI/*Xho*I fragment containing the entire mZP3 cDNA insert in plasmid pZP3 was transferred into the *Bam*HI/*Xho*I sites of the pFastbac HTb baculovirus expression vector (Gibco BRL, Gaithersburg, MD). The recombinant baculovirus expressing 6 × His-tagged mZP3 (bmZP3) was generated in Sf9 fall armyworm (*Spodoptera frugiperda*) pupal ovarian tissue insect cells (ATCC

CRL-1711) following standard transfection procedures according to the manufacturer's recommended protocol. Briefly, Sf9 cells grown in SF900II medium (Gibco BRL) at 1×10^6 ml⁻¹ were infected at a multiplicity of infection of 0.1 and grown at 29°C for 72–84 h in 100 ml suspension cultures (110 r.p.m.). The cells were harvested by centrifugation for 10 min at 1500 *g*, washed in PBS (137 mmol NaCl l⁻¹, 20 mmol Na₂HPO₄ l⁻¹, 1.5 mmol KH₂PO₄ l⁻¹, 2.5 mmol KCl l⁻¹, pH 7.4) and resuspended at 5×10^7 ml⁻¹. The cells were ruptured by sonication and the suspension was centrifuged at 3000 *g* for 10 min. The pelleted material was resuspended in PBS and heated in the presence of SDS, centrifuged at 10 000 *g* for 10 min and the resulting supernatant was used for the subsequent purification of bmZP3 using Ni-NTA Superflow resin (Qiagen, Clifton Hill, Victoria), according to the manufacturer's instructions, in the presence of 0.05% SDS. The eluted protein was dialysed against PBS and stored at –20°C until required for immunizations.

Expression and purification of recombinant mZP3 using vaccinia virus

Mouse ZP3 was cloned into the eukaryotic vaccinia virus–T7 RNA polymerase hybrid expression system using the vector pTM1 (Wyatt *et al.*, 1995). A DNA linker was inserted into the *Alw*NI site of mZP3 cDNA from plasmid pZP3 (Jackson *et al.*, 1998) to produce pTM1-mZP3 containing a 6 × His epitope inserted two amino acids downstream of the signal sequence. Recombinant vaccinia viruses were produced by transfection of vaccinia virus strain WR infected CV-1 African Green Monkey (*Ceropithecus aethiops*) kidney fibroblast cells (ATCC CCL70) using pTM1-mZP3. Recombinant vaccinia virus expressing mZP3 (vmZP3) was selected on 143B human fibroblast cells (ATCC CRL8303) according to standard procedures (Earl and Moss, 1997). Briefly, CV-1 kidney fibroblast cells were grown in minimal essential medium (MEM) supplemented with 10% newborn calf serum. All cells were grown in 5% CO₂ atmosphere at 37°C until confluent. The medium was then replaced with MEM without supplements and the cells were infected with recombinant vaccinia virus vmZP3 and helper virus vTF7-3 (Fuerst *et al.*, 1986) at a multiplicity of infection of one. Cells were incubated at 37°C in 5% CO₂ atmosphere for a further 60–72 h. The infected cells were detached from the flask using a cell scraper and poured into sterile 50 ml tubes. Infected cells were lysed by addition of Triton X100 to 1% v/v and the extract was centrifuged for 10 min at 3000 r.p.m. at room temperature (21°C). Supernatants were frozen at –20°C before protein purification. A wheat germ lectin Sepharose 6B column (Amersham International, Amersham) was equilibrated with five volumes of binding buffer (10 mmol Tris–HCl l⁻¹, pH 7.4). Thawed supernatant was

loaded at 0.5 ml per min and washed using 10 column volumes of binding buffer. Mouse ZP3 was eluted by applying two column volumes of elution buffer (0.1 mol *N*-acetyl glucosamine l^{-1} , 0.5% sodium deoxycholate, 10 mmol Tris-HCl l^{-1} , pH 7.4). The eluate was then loaded onto a Ni-NTA Superflow (Qiagen) column equilibrated with wash buffer (50 mmol Na_2HPO_4 l^{-1} , pH 8.0) and eluted using two column volumes of elution buffer (0.05% SDS, 50 mmol NaH_2PO_4 l^{-1} , pH 5.8). The samples were then dialysed against distilled water and lyophilized.

Mouse strains

BALB/c mice and wild-derived mice (SPFwd mice) were obtained from the Animal Resources Centre, University of Western Australia. SPFwd mice were specific pathogen-free mice randomly bred from offspring derived by Caesarian delivery from wild caught mice. The Animal Experimentation and Ethics Committee of the CSIRO Division of Sustainable Ecosystems approved all animal experimental procedures involving recombinant proteins, in compliance with the Australian National Health and Medical Research Council guidelines.

Immunization protocols

Female BALB/c mice ($n=61$), aged 6–8 weeks, were immunized and boosted three times either with bmZP3 ($n=20$), vmZP3 ($n=18$) or PBS ($n=23$). In the same way, female SPFwd mice ($n=20$) were immunized and boosted three times using Freund's adjuvants with bmZP3 ($n=10$) or PBS ($n=10$). The details of the immunizations are as follows. Mice immunized with bmZP3 or vmZP3 received an initial i.p. injection of 100 μl containing 20 μg antigen in 50 μl PBS mixed with 50 μl Freund's complete adjuvant (FCA; Sigma, St Louis, MO). Control mice received an initial i.p. injection of 100 μl containing 50 μl PBS without antigen mixed with 50 μl FCA. The animals were then boosted three times by i.p. injection of 100 μl containing either 20 μg antigen in 50 μl PBS mixed with 50 μl Freund's incomplete adjuvant (FIA) (treated groups) or 100 μl containing 50 μl PBS mixed with 50 μl FIA (control groups) at intervals of 2 weeks. Mice were bled from the suborbital sinus immediately before the first injection and again 2 (short-term trials) or 6 weeks (long-term trials) after the final boost. The blood was then centrifuged at 1500 r.p.m. for 5 min and the sera collected and stored at -20°C until tested. Mice were allocated to one of several fertility trials (see below) according to their strain and immunization type.

Short-term fertility trials with bmZP3 and vmZP3

Two control groups of BALB/c mice immunized with PBS only (groups B1, $n=5$ and group B3, $n=10$), two

groups of BALB/c mice immunized with bmZP3 (groups B2, $n=10$ and B4, $n=10$), and one group of BALB/c mice immunized with vmZP3 (group B5, $n=10$) were coupled 2 weeks after the final immunization with males of similar age (14–16 weeks old). Males were removed from all females after 2 weeks. All males used in these experiments were proved previously to be fertile by mating to untreated females. Female BALB/c mice in group B1 (control), group B2 (bmZP3) and group B5 (vmZP3) were allowed to produce litters. Female BALB/c mice in groups B3 (control) and B4 (bmZP3) were autopsied before they produced litters (around 17 days after mating) and viable embryos and failed implantation sites were counted.

One control group of SPFwd mice immunized with PBS only (group W1, $n=10$) and one group of SPFwd mice immunized with bmZP3 (groups W2, $n=10$) were coupled 2 weeks after the final immunization with single males of proven fertility of similar age (14–16 weeks old). Males were removed from all females after 2 weeks. Females were autopsied and viable embryos and failed implantation sites counted as for BALB/c mice in groups B3 and B4.

Long-term fertility trials with vmZP3

One control group of BALB/c mice immunized with PBS only (group B6, $n=8$) and one group of BALB/c mice immunized with vmZP3 (group B7, $n=8$) were coupled for the first time 6 weeks after the final immunization with single males of proven fertility of similar age (18–20 weeks old). Males were removed from all females after 2 weeks. After all mice had produced litters, females in groups B6 and B7 were again mated 14 weeks after the final immunization with single males of proven fertility of similar age (26–28 weeks old) and allowed to produce litters to determine long-term effects of vmZP3 on fertility.

Assay of antibodies

Antibody titres were determined by ELISA in 96-well microtitre plates (Greiner, Labortechnik GmbH, Frickenhausen). Briefly, antigens (ZP3 peptide, bmZP3 and vmZP3) were suspended in binding buffer (50 mmol NaHCO_3 l^{-1} , pH 9.6) and added to microtitre plates (0.5 μg antigen in 50 μl per well) and either incubated for 1 h at 37°C or overnight at 4°C . After the incubation, wells were washed three times with ELISA buffer (145 mmol NaCl l^{-1} , 7.5 mmol Na_2HPO_4 l^{-1} , 2.5 mmol NaH_2PO_4 l^{-1} , containing 0.005% Tween-20, pH 7.4). Non-specific binding was prevented by incubation in ELISA buffer containing 5% (w/v) skim milk powder for 1 h at 37°C . Plates were washed three times with ELISA buffer and 50 μl of serum samples serially diluted in ELISA buffer containing 1% (w/v) skim milk powder were added to wells in duplicate and

incubated for 1 h at 37°C. Wells were washed three times with ELISA buffer and 50 µl secondary antibody (affinity purified and horseradish peroxidase-labelled goat anti-mouse IgG (Silenus Laboratories, Hawthorn, Victoria)) diluted 1:3000 in ELISA buffer containing 1% (w/v) skim milk powder was added to each well. After a further incubation of 1 h at 37°C, plates were washed with ELISA buffer and colour was developed by adding 50 µl development buffer. Development buffer (0.01% H₂O₂, 2 mmol ABTS l⁻¹ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Sigma) in acetate buffer (100 mmol sodium acetate l⁻¹, 50 mmol NaH₂PO₄ l⁻¹, pH 4.0)) was prepared immediately before use. Reactions were terminated after 20 min by addition of 50 µl of 10% SDS and absorbance was measured at 405 nm on an ELISA plate reader (Bio-Rad, Hercules, CA). Means of duplicate samples are reported. In addition, other microtitre plates including MaxiSorp and PolySorp (Nalge Nunc, Rochester, NY), Carbo-bind (Corning Inc., Corning, NY), Immulon 2HB (Dynex Technologies, Chantilly, VA) and Ni-NTA (Qiagen) were tested for suitability in ELISAs with vmZP3 protein using various binding and detection buffers recommended by the manufacturers. Bound primary antibody was detected using affinity purified and horseradish peroxidase-labelled goat anti-mouse IgG (Silenus Laboratories) diluted 1:3000.

Western blotting

Proteins were denatured by heating to 95°C for 5 min in loading buffer under reducing conditions (10% (w/v) glycerol, 2% (w/v) SDS, 0.0025% (w/v) bromophenol blue, 5% β-mercaptoethanol, 65 mmol Tris-HCl l⁻¹, pH 6.8) and separated in 12% discontinuous SDS-polyacrylamide gels (Laemmli, 1970). Proteins were transferred to PVDF membranes (NEN Life Science Products, Boston, MA) by electrophoretic transfer (Mini Trans-Blot; Bio-Rad) and used for immunoblot analysis of mouse sera (Towbin *et al.*, 1979). PVDF membranes were blocked overnight at 4°C in TBS buffer (150 mmol NaCl l⁻¹, 10 mmol Tris-HCl l⁻¹, pH 8.0) containing 5% skim milk powder. Membranes were rinsed and incubated at room temperature for 1 h with primary antibodies diluted in TBS containing 1% skim milk powder. Unbound primary antibody was removed by three washes in TBS containing 0.05% Tween-20 and membranes were incubated at room temperature for 1 h with secondary antibodies diluted in TBS containing 1% skim milk powder. Membranes were washed with TBS and bound secondary antibodies were visualized by immersion of the immunoblot in detection buffer (PBS buffer containing 0.05% (w/v) 3,3'-diaminobenzidine and 0.003% H₂O₂) until bands became visible. Control primary antibodies were polyclonal rabbit anti-mouse ZP3 sera raised in rabbits infected with recombinant myxoma virus expressing mouse ZP3 (Jackson *et al.*,

1998) and polyclonal sera raised in rabbits against whole pig zona pellucida (a gift from M. Bradley, Vertebrate BioControl CRC, Canberra). Affinity purified and horseradish peroxidase-labelled goat anti-mouse IgG or goat anti-rabbit IgG (Silenus Laboratories) were diluted 1:3000 and used for detection of sera from mice or rabbits, respectively.

Statistical analysis

Results are presented as means ± SEM. Data were analysed by one-way ANOVA and multiple comparisons between groups were conducted using the Tukey-Kramer multiple comparison test (Number Cruncher Statistical System, NCS, Kaysville, UT). A value of *P* < 0.05 was accepted as significant.

Results

Production of recombinant mZP3 proteins

The predicted size of the recombinant bmZP3 protein (coupled to a 6 × His tag) was 452 amino acids with an estimated molecular mass without post-translational modification of 49.7 kDa (ExPASy translate tool, Swiss Institute of Bioinformatics; Appel *et al.*, 1994). Recombinant bmZP3 stained with Coomassie blue appeared as two or three bands with apparent molecular masses of between 45 and 55 kDa in SDS-PAGE gels under reducing conditions (Fig. 1).

The predicted size of the precursor recombinant vmZP3 protein (coupled to a 6 × His tag) was 430 amino acids with an estimated molecular mass without glycosylation or post-translational modification of 47.1 kDa. Recombinant vmZP3 stained with Coomassie blue appeared as two double bands with apparent molecular masses of about 55 and 60 kDa in SDS-PAGE gels under reducing conditions (Fig. 1).

Both recombinant bmZP3 and vmZP3 proteins were recognized on western blots by polyclonal sera produced in rabbits against whole pig zona pellucida. Under these conditions, bmZP3 appeared as two broad bands with apparent molecular masses of 45 and 55 kDa, whereas vmZP3 appeared as two broad bands with apparent molecular masses of 55 and 60 kDa (Fig. 1). Similar molecular masses were obtained when vmZP3 was probed on western blots using sera raised in rabbits infected with recombinant myxoma virus expressing mZP3 (Fig. 2a, lane 13).

Antibody responses of mice versus recombinant bmZP3 protein

ELISA was used to assess the antibody responses of the mice immunized with recombinant bmZP3 or vmZP3 (Table 1). Sixteen of the 20 BALB/c mice that received bmZP3 injections (groups B2 and B4) had antibody

Fig. 1. SDS-PAGE and western blot analysis of affinity purified recombinant bmZP3 (mouse zona pellucida 3 produced in a baculovirus insect cell-expression system) and vmZP3 (mouse ZP3 produced in a vaccinia virus mammalian cell-expression system) proteins. Samples (1 μ g) of each protein were separated on a SDS-PAGE gel under reducing conditions and either stained with Coomassie blue or transferred to PVDF membranes and probed with polyclonal rabbit anti-whole pig zona pellucida sera raised in rabbits, diluted 1 : 1000. Secondary antibodies were horseradish peroxidase-labelled goat anti-rabbit IgG. Lane 1: bmZP3 protein stained with Coomassie blue; lane 2: vmZP3 protein stained with Coomassie blue; lane 3: bmZP3 protein probed with pig zona pellucida sera; lane 4: vmZP3 protein probed with pig zona pellucida sera; lane 5: low range molecular weight markers.

responses to bmZP3 that were above background cut-off levels (absorbance = 0.1). At serum dilutions of 1 : 100, absorbance values ranged from < 0.1 to 2.16 with a mean of 0.35 ± 0.11 . Four BALB/c mice also had antibody responses to bmZP3 at serum dilutions of 1 : 1000. Mean antibody responses against bmZP3 protein were three-fold higher in SPFwd mice than in BALB/c mice. All ten SPFwd mice that received bmZP3 injections (group W2) had antibody responses to bmZP3 above background levels (absorbance > 0.1). Nine of these also had antibody responses at serum dilutions of 1 : 1000. At serum dilutions of 1 : 100, absorbance values ranged from 0.20 to 2.41 with a mean of 1.51 ± 0.21 , and at 1 : 1000 serum dilution values ranged from < 0.1 to 1.47 with a mean of 0.48 ± 0.14 . Only one vmZP3-immunized mouse (from group B5) produced an absorbance response (0.11) to bmZP3. Antibody titres were below background cut-off levels (absorbance = 0.1) for all pre-immune sera and all sera from control BALB/c

and wild mice (groups B1, B3, B6 and W1) at 1 : 100 serum dilutions.

Antibody responses of mice versus recombinant vmZP3 protein

ELISA failed to detect vmZP3-specific antibodies versus the vmZP3 antigen, but western blot analysis identified them readily. None of the vmZP3 immunized mice produced detectable IgG antibody titres by ELISA at serum dilutions of 1 : 100 against the vmZP3 protein (data not shown). Sera from mice immunized with the mammalian cell-expressed ZP3 protein failed to detect purified vmZP3 protein added to a range of microtitre plates from several different suppliers. In contrast, all sera collected 2 weeks after immunizations were completed (from vmZP3 immunized mice in group B5) contained IgG antibodies that crossreacted strongly to vmZP3 on western blots (lanes 1–10, Fig. 2a). Only two of seven sera collected 6 weeks after immunizations with vmZP3 were completed (group B7) contained IgG antibodies that crossreacted weakly to vmZP3 on western blots (lanes 3–4, Fig. 2b). Sera from BALB/c mice immunized with bmZP3 collected two weeks after immunizations were completed crossreacted only weakly to vmZP3 on western blots (lanes 11–12, Fig. 2a). No crossreaction was detected for pre-immune or control sera to vmZP3 on western blots at serum dilutions of 1 : 100 (result not shown).

Antibody responses of mice versus mZP3 peptide

Only five mice had detectable antibody responses when tested by ELISA against the mZP3 peptide at serum dilutions of 1 : 100. These mice were also the highest responders to bmZP3 from their respective groups. These included four mice immunized with bmZP3 (two infertile BALB/c in group B2 and two fertile wild mice in group W2) and a single mouse immunized with vmZP3 (an infertile BALB/c mouse in group B5). Specifically, absorbance responses to the ZP3 peptide were 0.41 and 0.58 (group B2), 0.81 and 0.58 (group W2) and 0.18 (group B5). The mouse in group B5 was the only mouse to produce an absorbance response to the bmZP3 protein.

Fertility of BALB/c mice immunized with recombinant bmZP3 protein

There was no significant reduction in the fertility or mean litter sizes of BALB/c mice immunized with the recombinant bmZP3 protein (Table 2). Data for the viable embryos and pups born in groups B1 and B3 (controls) were pooled for statistical analyses, as were the data for groups B2 and B4 (bmZP3), as control group B1 contained only five mice and the immunization regimens were identical. Fifteen of the 20 BALB/c mice treated with

Table 1. Antibody response by ELISA of mice immunized with recombinant mouse zona pellucida 3 (ZP3) proteins

Group	Mouse strain	Antigen	Sera collected	Weeks after 3rd boost	Mean ($\pm$ SE) absorbance 1 : 100	Number of responders ^a
B1	BALB/c	PBS	Final	2	0	0/5
B3		PBS	Final	2	0.07 $\pm$ 0.01	0/10
B6		PBS	Final	6	0.01 $\pm$ 0.01	0/8
B2		BmZP3	Pre-immune	–	0.03 $\pm$ 0.01	–
B2		BmZP3	Final	2	0.43 $\pm$ 0.22	6/10
B4		BmZP3	Pre-immune	–	0.03 $\pm$ 0.01	–
B4		BmZP3	Final	2	0.26 $\pm$ 0.04	10/10
B5		VmZP3	Pre-immune	–	0	–
B5		VmZP3	Final	2	0.03 $\pm$ 0.01	1/10
B7		VmZP3	Pre-immune	–	0	–
B7	SPFwd	VmZP3	Final	6	0.03 $\pm$ 0.01	0/8
W1		PBS	Final	2	0.02 $\pm$ 0.01	0/10
W2		BmZP3	Pre-immune	–	0.02 $\pm$ 0.01	–
W2		BmZP3	Final	2	1.51 $\pm$ 0.21	10/10

bmZP3: mouse ZP3 produced in a baculovirus insect cell-expression system; vmZP3: mouse ZP3 produced in a vaccinia virus mammalian cell-expression system.

^aNumber of mice considered to have detectable serum antibodies (absorbance > 0.1) to bmZP3 protein by ELISA relative to the total number of mice at 1 : 100 serum dilutions.

Fig. 2. Western blot analysis of sera from BALB/c mice immunized with recombinant mouse zona pellucida 3 (mZP3) proteins. Sera from mice immunized and boosted three times with vmZP3 (mouse ZP3 produced in a vaccinia virus mammalian cell-expression system) or bmZP3 (mouse ZP3 produced in a baculovirus insect cell-expression system) were diluted 1:100 and used to probe vmZP3 protein that was separated on a SDS-PAGE gel under reducing conditions and transferred to PVDF membranes. Secondary antibodies were horseradish peroxidase-labelled goat anti-mouse IgG. (a) Sera from mice 2 weeks after the final boost. Lanes 1–2: vmZP3 immunized fertile mice from group B5 (BALB/c mice immunized with vmZP3); lanes 3–10: vmZP3 immunized infertile mice from group B5; lane 11: a bmZP3 immunized infertile mouse from group B2 (BALB/c mice immunized with bmZP3); lane 12: a bmZP3 immunized fertile mouse from group B2; lane 13: sera from a rabbit immunized with mZP3 produced by recombinant myxoma virus. (b) Sera from mice 6 weeks after the final boost. Lane 1: PBS immunized mouse from group B6 (BALB/c mice immunized with PBS only); lanes 2–8: vmZP3 immunized fertile mice from group B7 (BALB/c mice immunized with vmZP3).

bmZP3 (groups B2 and B4) were fertile compared with 12 of 15 in the controls (groups B1 and B3). Mean litter sizes of the bmZP3 immunized (4.7 ± 0.8) and control (5.0 ± 0.9) mice were not significantly different ($P = 0.64$). The two BALB/c animals (from group B2) that produced

antibodies to the ZP3 peptide by ELISA at 1 : 100 serum dilutions were infertile, although the two wild mice (from group W2) with similar responses to the ZP3 peptide antibodies were fertile. Maximum litter size was 13 for BALB/c mice in these experiments.

Table 2. Fertility of mice immunized with recombinant mouse zona pellucida 3 proteins

Group	Mouse strain	Antigen	Mating (weeks after 3rd boost)	Number of fertile mice	Total number of pups	Total number of embryos	Failed implants	Mean ($\pm$ SE) litter size of mice	P
B1	BALB/c	PBS	2	2/5	10			2.0 $\pm$ 1.6	
B2		bmZP3	2	8/10	50			5.0 $\pm$ 1.1	
B3		PBS	2	10/10	–	65	2	6.5 $\pm$ 1.1	
B4		bmZP3	2	7/10	–	44	2	4.4 $\pm$ 1.1	
B1 + B3		PBS	2	12/15	75			5.0 $\pm$ 0.9	
B2 + B4		bmZP3	2	15/20	94			4.7 $\pm$ 0.8	0.64 ^c
B5		vmZP3	2	2/10	5			0.5 $\pm$ 0.4	0.0015 ^c
B6	SPFwd	PBS	6	8/8	43			5.4 $\pm$ 0.6	
B7		vmZP3	6	7/7 ^a	29			4.1 $\pm$ 0.8	0.38
B6		PBS	14	7/7 ^a	58			8.3 $\pm$ 0.4	
B7		vmZP3	14	7/7 ^a	23			3.3 $\pm$ 0.6	0.017
W1		PBS	2	9/10	–	43	9	4.3 $\pm$ 0.8	
W2		bmZP3	2	9/9 ^a	–	57	2	6.3 $\pm$ 0.5	0.11

bmZP3: mouse ZP3 produced in a baculovirus insect cell-expression system; vmZP3: mouse ZP3 produced in a vaccinia virus mammalian cell-expression system.

P values were obtained using one-way ANOVA and indicate where significant difference ($P < 0.05$) in litter size was present between treated and control groups.

^aOne female died during treatment.

^bMice were autopsied and viable embryos were counted.

^cSignificance values were determined relative to pooled group B1 + B3 PBS controls.

Fertility of SPFwd mice immunized with recombinant bmZP3 protein

There was no significant reduction in the fertility or mean litter sizes of female SPFwd mice immunized with the recombinant bmZP3 protein (Table 2) compared with controls. All nine bmZP3 immunized female SPFwd mice (group W2) were fertile compared with nine of ten SPFwd control mice (group W1). Mean litter sizes of the treated (6.3 ± 0.5) and control (4.3 ± 0.8) mice were not significantly different ($P = 0.11$). There were more implantation failures in the SPFwd mice than in the BALB/c mice, as indicated by the presence of visible embryo resorption sites (11 resorptions from one treated and five control mice), than in the BALB/c mice (four resorptions from two treated and two control mice). This difference was due to a single control SPFwd mouse that had six implantation failures. Maximum litter size was nine for SPFwd mice in these experiments.

Fertility of BALB/c mice mated 2 weeks after immunization with recombinant vmZP3 protein

There was a 75% reduction in the fertility of mice immunized with vmZP3 and mated 2 weeks after the final immunization (group B5) relative to the controls (groups B1 and B3; Table 2). Only two out of ten (20%) vmZP3 immunized mice were fertile (group B5), compared with 12 of 15 (80%) of the controls (groups B1 and B3). Mean litter size of the vmZP3 immunized mice (0.5 ± 0.4) was significantly different ($P = 0.0015$), and was reduced to 10% of the controls (5.0 ± 0.9). A Tukey-Kramer multiple comparison test was used to test

for all pair-wise differences between the mean litter sizes of all groups of BALB/c and wild mice immunized with vmZP3, bmZP3 or PBS and mated 2 weeks after the final boosts. Only group B5 (vmZP3 immunized) was found to differ significantly from other treated and control groups (B2, B3, B4, B6 and W2; $F(8, 70) = 4.57$, $P = 0.00017$).

Fertility of BALB/c mice mated 6 and 14 weeks after immunization with recombinant vmZP3 protein

There was no significant difference in fertility and mean litter size of mice immunized with vmZP3 and mated for the first time 6 weeks after the final boost (group B7) compared with controls (group B6). All mice were fertile, and although mean litter size in the vmZP3 immunized group (4.1 ± 0.8) was only 76% of the controls (5.4 ± 0.6), this was not significantly different ($P = 0.38$). Mice in these two groups were re-mated 14 weeks after the final boost and all seven mice in groups B6 (controls) and B7 (vmZP3) were still fertile. However, the mean litter size of vmZP3 immunized mice at the second mating (3.3 ± 0.6) was significantly different ($P = 0.017$), and was reduced to 40% of the litter size of the re-mated controls (8.3 ± 0.4).

Discussion

Direct immunization of BALB/c mice with vmZP3 produced in a mammalian expression system leads to production of vmZP3-specific antibodies and reduction in fertility. In one group of BALB/c mice, fertility and fecundity were reduced markedly compared with those

of control animals. Fertility was reduced to 25% and pup production to 10% of controls when the mice were mated 2 weeks after receiving an immunization regimen with vmZP3 in Freund's adjuvants, but the effect on fertility was only transient. In a second group of BALB/c mice, mouse-ZP3 specific antibodies were barely detectable and all mice were fertile when they were mated both 6 and 14 weeks after immunization with vmZP3. However, pup production was reduced in this second group to 76% and 40% of controls at 6 weeks and 14 weeks, respectively. These results demonstrate that transient immune responses and infertility can be induced in BALB/c mice immunized with recombinant mZP3 protein produced in a mammalian cell-expression system, and that there are potentially long-term effects on litter production.

In contrast, no effect on either fertility or fecundity of BALB/c mice or SPFwd mice could be demonstrated in mice immunized with bmZP3 produced in an insect cell-expression system, despite the presence of bmZP3-specific antibodies. SPFwd mice, in addition to BALB/c mice, were tested with bmZP3 protein because previous studies have shown that specific pathogen-free wild mice respond more strongly to mZP3 peptide antigens than did BALB/c mice (Hardy *et al.*, 2002). Indeed, SPFwd mice did produce higher bmZP3-specific antibodies than did BALB/c mice, although there was no effect on fertility in either strain.

Although some similarities in biochemical and immunological properties were present between the recombinant bmZP3 and vmZP3 proteins, several observations support the conclusion that the contraceptive effect of vmZP3 was due to immune responses by the mice to epitopes provided by expression of the mZP3 in a mammalian but not an insect cell line. Insect and mammalian cells are both able to produce *N*-linked glycosylated proteins, but the glycosylation patterns differ (Altmann *et al.*, 1999). More importantly, insect cells do not normally provide *O*-linked glycosylation (Marchal *et al.*, 2001). These differences have important implications in the source of recombinant mZP3 antigens to be used for inducing immunological blocks to fertility, as binding of primary spermatozoa to the mZP3 subunit is known to involve *O*-linked oligosaccharide residues (Bleil and Wassarman, 1980b; Florman and Wassarman, 1985; Bleil and Wassarman, 1988; Miller *et al.*, 1992; Thaler and Cardullo, 1996). Certain critical immuno-contraceptive epitopes are likely to have been provided by *N*- and *O*-linked oligosaccharides after processing of the mZP3 in mammalian but not in insect cells, and as described below, this conclusion is supported by differences observed in the apparent molecular masses, immunogenicity and chemical properties of the bmZP3 and vmZP3 proteins.

First, the apparent molecular mass of recombinant vmZP3 (55–60 kDa) was greater than that of bmZP3 (45–55 kDa), although both were considerably less than those

reported for native mZP3 (83 kDa), but equal or greater than predicted from their amino acid sequences (47–50 kDa). The size of the vmZP3 was more similar than that of bmZP3 to those previously reported (60–83 kDa) for other recombinant forms of mZP3 known to contain both *N*- and *O*-linked glycosylated residues following expression in mammalian cells (Kinloch *et al.*, 1991; Beebe *et al.*, 1992; Liu *et al.*, 1997; Chen *et al.*, 1998). This finding is consistent with greater glycosylation on vmZP3 than on bmZP3. In addition, all preparations of recombinant bmZP3 and vmZP3 showed at least two well-separated bands with different molecular masses on SDS-PAGE gels under denaturing conditions. This result again indicates the presence of different post-translationally modified forms of the two proteins.

Second, both the recombinant bmZP3 and vmZP3 proteins were detected on immunoblots using rabbit antisera raised against whole pig zona zellucida. This was despite the fact that the amino acid sequence conservation between the mouse and pig ZP3 proteins and homology is less than 66% (Harris *et al.*, 1994). In contrast, there was only very weak, immunological crossreaction of sera from BALB/c mice immunized with bmZP3 or vmZP3 to the vmZP3 and bmZP3 proteins, respectively. This was true for both immunoblots, where strong denaturation of the proteins (SDS, reducing and boiling) might expose, destroy or modify peptide epitopes, or for ELISAs under non-denaturing conditions, where conformational epitopes are retained. This result showed that mice recognized different epitopes on vmZP3 and bmZP3, even though the amino acid sequences of the two recombinant forms of protein differed only in the location of the short 6 × His tag. Some mice did produce low titres of antibodies to a mouse ZP3 peptide (CSNSSSSQFQIHGPR) after immunization with bmZP3 or vmZP3, indicating that this amino acid sequence was recognized from both recombinant proteins by individual animals. It has previously been reported that infertility in mice is directly associated with antibody titres to this peptide (Millar *et al.*, 1989; Lou *et al.*, 1995; Hardy *et al.*, 2002). However, there was no evidence of any correlation between the presence of ZP3 peptide-specific antibodies and infertility in mice immunized with either the bmZP3 or vmZP3 proteins.

Finally, differences in post-translational modifications such as glycosylation could explain the ability in the present study to develop an ELISA assay using the purified bmZP3, but not vmZP3, protein. Either the larger and more heavily glycosylated vmZP3 protein did not bind to microtitre plates, or immunodominant epitopes of vmZP3 but not of bmZP3 were masked. Interestingly, a crude preparation of mZP3 protein produced in myxoma virus has previously been shown to be effective as a detection antigen in ELISAs for sera from animals exposed to mZP3 antigen produced in ectromelia virus (Jackson *et al.*, 1998) and murine cytomegalovirus (Lloyd *et al.*, 2003). In these cases, mZP3 protein was able to bind

to the plate in cell debris present in the crude antigen preparations. This antigen source was not suitable in ELISA for animals injected with vmZP3 due to an unacceptably high background in the assay. These results all support the conclusion that vmZP3 and bmZP3 were subject to different levels of post-translational modification, and that contraceptive antibody responses to vmZP3 were induced in the mice against glycosylated epitopes rather than against peptide sequences.

It is possible that factors other than the presence of appropriate glycosylation were responsible for the ability of vmZP3 but not bmZP3 to block fertility in immunized mice. Protein construction or purification protocols may have denatured the bmZP3 protein and reduced immunogenicity, whereas those used to purify the vmZP3 protein did not. For example, the 6×His tag was attached upstream of the 22 amino acid signal sequence in the bmZP3 protein rather than after the signal sequence, as was the case for the vmZP3 protein. As a consequence, this could have either interfered with post-translational modifications such as protease cleavage in the bmZP3 protein or generated novel immunogenic peptide epitopes for vmZP3 but not bmZP3. Likewise, protein purification protocols may have denatured the bmZP3 protein and reduced immunogenicity, whereas those used to purify the vmZP3 protein did not. However, these explanations are not supported by other observations. For example, both proteins were exposed to SDS during the purification procedures and as vmZP3 was effective, exposure to denaturing agents does not appear to prevent contraceptive potential. In addition, it has been reported that recombinant human ZP proteins containing 6×His tags produced in insect cells are much less immunogenic than the same proteins with identical 6×His tags produced in mammalian cells (Harris *et al.*, 1999).

It has been proposed that the cellular and cytokine responses to immunization with ZP antigens are critical in determining the eventual fertility status of individual animals through processes that lead to oophoritis (Ramsay and Ramshaw, 1997; Lou *et al.*, 2000). It is not possible here to assess the role of cellular immunity in the induction of infertility of mice immunized with recombinant mZP3s, although there is some evidence that cellular factors influenced the immune responses of mice to bmZP3 and vmZP3. In particular, SPFwd mice produced generally higher antibody responses to bmZP3 than did BALB/c mice. This is likely to be due to greater cellular immune responses in SPFwd mice from increased variation in genetic factors such as the major histocompatibility complex molecules of these outbred mice compared with inbred BALB/c mice. Second, two fertile vmZP3-immunized BALB/c mice had antisera that crossreacted strongly to vmZP3 on immunoblots 2 weeks after immunization, whereas all other mice with antibodies were infertile. This finding indicates that infertility may require factors other than antibodies.

However, as vmZP3 antibodies in other mice were barely detectable by 6 weeks after immunization and all were fertile, it is also possible that the concentrations of vmZP3 antibodies in the two mice were below those able to cause infertility. This could not be confirmed in the present study, as the assay system for vmZP3 was not quantitative.

Delivery of antigens remains one of the most challenging requirements for the development of effective immunocontraceptive vaccines. Almost all studies aimed at contraception using recombinant proteins or peptides have relied on direct injection and multiple boosts of antigen to achieve their effect. Most require high titres of specific antibodies, and rapid return of fertility occurs once immunization regimens are halted. The present study has confirmed that purified recombinant proteins are able to induce infertility when produced in a suitable expression system, but that the effects are transient. The results of the present data support the contention that achieving effective infertility in mice using mZP3 antigens requires the use of recombinant mammalian viruses such as murine cytomegalovirus (Chambers *et al.*, 1999; Lloyd *et al.*, 2003) or ectromelia virus (Jackson *et al.*, 1998) to deliver appropriately modified forms of the antigen. In addition, one of the most attractive features of this approach is that long-term infertility is obtained after only single inoculations of mice with such recombinant viruses, probably through provision of complex adjuvant effects (Jackson *et al.*, 1998; Lloyd *et al.*, 2003). The ability of viruses to predispose animals to autoimmune diseases (Schattner and Rager-Zisman, 1990; Overwijk *et al.*, 1999) makes it worth considering them as vaccine vectors for long-term contraception in the future.

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