Development of mouse-specific contraceptive vaccines: infertility in mice immunized with peptide and polyepitope antigens

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Abstract

Mouse-specific immunocontraceptive peptides have been identified in mouse proteins with key roles in reproduction from sequence comparisons to other species and tested for efficacy as immunocontraceptive antigens. Peptides were derived from granulocyte-macrophage colony-stimulating factor (GMCSF), the placental 27 kDa heat-shock protein (HSP), leukemia inhibitory factor receptor (LIFR), oviduct glycoprotein (OGP), proliferin (PLF), prolactin (PRL), sperm protein SP56 and mouse zona pellucida subunits 1 and 3 (ZP1, ZP3). Fertility of female BALB/c mice was reduced after immunization with several peptides either conjugated to a carrier protein or in the form of recombinant polyepitopes. The most effective conjugated peptides (SP56, GMCSF and PRL) induced peptide-specific serum antibodies and reduced fertility by 50%. Fertility of mice was also reduced after immunization with polyepitope antigens containing up to five different peptides fused to maltose-binding protein, but antibodies were not produced against all the encoded peptides. The most effective polyepitope antigen (containing PLF, SP56, ZP1 and ZP3 peptides) reduced fertility by 50% but induced only SP56 and ZP1 antibodies. We demonstrate that lack of antibody response to a given peptide epitope (ZP3) can be overcome if repeated copies are used in the polyepitope antigen construct, but the effect varies between mouse strains. We conclude that infertility induced in mice with a range of peptide-based vaccines is dependent on antigen formulation and genetic factors but does not necessarily correlate with peptide-specific antibody levels. In light of these results, strategies to improve the efficacy of peptide-based antifertility vaccines are discussed.

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Introduction

Contraceptive vaccines are being developed to prevent fertility by triggering an autoimmune response to molecules essential for reproduction. Immunization against various proteins in sperm, testis, ovary, hormones and their receptor proteins has been successful at reducing fertility in a wide range of species (Delves *et al.* 2002, Ferro 2002). Some of the most effective antifertility vaccines currently under development use genetically modified viruses to deliver the reproductive antigens. Specifically, recombinant mouse-specific viruses, such as ectromelia virus (ECTV) and murine cytomegalovirus (MCMV), expressing the entire mouse egg protein ZP3 have been shown to cause long-term infertility in infected mice (Jackson *et al.* 1998, Lloyd *et al.* 2003). The objective of this type of research is to produce transmissible vaccines that will spread in the field and cause sufficient infertility to reduce populations of the targeted animals to manageable levels (Tyndale-Biscoe 1994).

Contraceptive vaccines must be effective, long-lasting, cheap and readily applicable, and have no unacceptable side effects (Grandy & Rutberg 2002). They may or may not need to be reversible depending on the application (Hoskinson *et al.* 1990, Aitken 2002). However, an additional but critical requirement for infertility vaccines intended for broad-scale uses, such as wildlife population control, is that they must be species-specific (Bradley *et al.* 1999). Lack of species-specificity of antifertility vaccines is widely documented for a range of reproductive antigens from sperm (Bandivdekar *et al.* 1991, Naz 2000), egg (Barber & Fayer-Hosken 2000) and hormones such as LHRH and human chorionic gonadotrophin (hCG) (Talwar 1997). Several groups have even identified universal contraceptive peptide antigens by immunologic or sequence-based comparisons of antigens derived from sperm (Zhu & Naz 1994, O'Hern *et al.* 1995), egg (Hall *et al.* 1995, Zhu & Naz 1999, Carino *et al.* 2002), hormones (Talwar 1997) and other proteins (Subramanian *et al.* 2003). Therefore,

reliance on the species-specificity of the delivery vectors in the many vaccines under development raises the possibility that the fertility of non-target species could inadvertently be affected.

Immunization with proteins can have unexpected and undesirable side effects, including autoimmune diseases, particularly with self-antigens that have multiple functions or show considerable sequence homology to other proteins (Nettles 1997). To reduce the possibility of unwanted side effects and increase specificity, targeting of functional regions of the antigens is an attractive approach. Contraceptive peptide epitopes with reduced side effects (Lou *et al.* 1995, Sun *et al.* 1999) and increased species-specificity (Hinsch *et al.* 1999) have been described. These results show that peptide antigens can increase the species-specificity of antifertility vaccines, with the added benefit of reducing side effects.

The design of antifertility vaccines must address many of the same challenges facing the development of anticancer vaccines. Specifically, both types of vaccine should not affect normal tissues or their function, be able to overcome host down-regulation of autoreactive immune cells, and be efficacious in as many individuals as possible to reduce development of genetic resistance (Ferro 2002). In order to address these issues, anticancer vaccines under development use multiple peptides (Valmori *et al.* 2003) or polyepitope and multiple antigen peptides that contain strings of tumor-derived peptide epitopes and enhancers (Smith 1999, Suhrbier 2002). A similar need for multiple peptide antigens and immune enhancers in antifertility vaccines is demonstrated by the fact that individual peptides are often ineffective at inducing cross-reactive immunity to the native protein targets, perhaps for reasons such as conformational difference between the peptide and native protein or the inability of individuals to recognize certain epitopes due to major histocompatibility complex (MHC) restriction (Lou *et al.* 1995, Lea *et al.* 1998). In addition, polyepitope vaccines can potentially more closely mimic cases of documented natural autoimmunity where the physiological effect appears to require simultaneous immune responses against a number of different antigenic molecules (Bach *et al.* 1998).

The need for mouse-specific contraceptive antigens (Bradley *et al.* 1999), coupled with the availability of a wide range of reproductive proteins in the mouse genome database, makes this an ideal species in which to identify species-specific contraceptive peptides. Once promising peptides have been identified, they could potentially be used in combination with immune enhancers (Ramsay & Ramshaw 1997) in the form of multivalent or polyepitope vaccines to generate stronger immune responses and more effective infertility. We have previously shown that the fertility of mice immunized with polyepitope antigens containing combinations of existing and novel mouse-specific peptides is significantly reduced (Hardy *et al.* 2002a).

Here, we report the relationship between infertility and the peptide-specific antibody responses in mice

immunized with polyepitopes, and compare these to the responses in mice immunized with individual mouse-specific peptides. On the basis of our results, we propose improvements in the design of species-specific polyepitope antigens that increase both immunogenicity and contraceptive effect.

Materials and Methods

Mouse-specific peptides

Mouse-specific peptides were derived from granulocyte-macrophage colony-stimulating factor (GMCSF), the placental 27 kDa heat-shock protein (HSP), leukemia inhibitory factor receptor (LIFR), oviduct glycoprotein (OGP), proliferin (PLF), prolactin (PRL), sperm protein SP56 and mouse zona pellucida subunits 1 and 3 (ZP1, ZP3). The peptide sequences were selected from protein regions with relatively low homology between mouse and other species such as man (Table 1). Eight of the peptides (GMCSF, HSP, LIFR, OGP, PLF, PRL, SP56 and ZP3) were chemically synthesized by Mimotopes Pty Ltd (Clayton, Australia) with N- or C-terminal cysteine residues, naturally present in some of the peptides (GMCSF, PLF, SP56 and ZP3), to facilitate cross-linking to carrier molecules (Table 1). Peptide sequences and purity were determined by the manufacturers using reverse-phase HPLC. Due to the relatively large size of the ZP1 peptide (102 amino acids), it was produced and bounded by naturally occurring cysteine residues within a 6XHis fusion protein in *Escherichia coli*.

Conjugation of peptides to KLH

Peptides for immunization (Table 1) were conjugated to the carrier protein keyhole limpet hemocyanin (KLH) (Sigma) through the cysteine of the peptide by using *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester (MBS) (Sigma) as the coupling reagent (Muller 1988). MBS (10 mg/ml in dimethylformamide) was added dropwise to a solution of KLH (10 mg/ml in 31 mM Na₃PO₄, 460 mM NaCl and 41 mM sucrose) to give a final molar ratio of MBS to KLH of 40:1 (14 µg MBS/mg KLH). Unreacted MBS was removed by filtration through a Sephadex G-25 M column (PD-10; Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM phosphate buffer, pH 6.0. Activated KLH carrier protein was mixed with peptide (2 mg/ml) dissolved in PBS, pH 7.4, to give a molar ratio of peptide:protein of 40:1 (0.2 mg peptide/mg KLH conjugate). The mixture was incubated for 3 h at room temperature with constant stirring and dialyzed against PBS overnight, and aliquots were stored at -20 °C.

Conjugation of peptides to BSA

Two peptides (GMCSF and HSP) that failed in the immunologic assays when used directly were conjugated to bovine serum albumin (BSA) with glutaraldehyde (Muller 1988) for use in ELISA. Specifically, peptide was added

Table 1 Peptides used to immunize mice. Sequences (sourced from the GenBank database) are shown in standard single-letter code.

Peptide	Gene	Mouse GenBank	Location (amino acids)	Peptide sequence	Minimum purity	Human GenBank	Human/mouse peptide sequence identity
GM-CSF	Granulocyte macrophage colony stimulating factor	X03019	56–68	H-VSNFEFSFKLTC-NH ₂	95%	M10663	38%
HSP	27 kDa placental heat-shock protein	U03560	64–73	H-CAEGLAVTLAA-OH	81%	X54079	50%
LIFR	Leukemia inhibitory factor receptor	D26177	114–126	H-CNGFQSKFTLINEKD-OH	95%	X61615	63%
OGP	Oviduct glycoprotein	D32137	529–550	H-CKATAGISKTPEISKATAGVSK-OH	90%	U09550	Nil
PLF	Proliferin	K02245	180–199	H-FLQSDNEDARIHSYGMISC-OH	19%	Not known	Not known
PRL	Prolactin	X02892	100–121	H-CLKVPPEVLNLLISLVQSSDP-OH	25%	X54393	19%
SP56	56 kDa sperm protein	U17108	438–454	H-CYLFGEENSTEAMKGO-OH	92%	Not known	Not known
ZP1 ^a	Zona pellucida subunit 1	U20448	127–228	CPKPDHTVTPDPYLAAPPTPEFTPHA FALHPIDHTLAGSGHTGLTLYPEQS FIHPTAPPSLCGPGAGSTVPHSQWGL EPWELTELDVSGTHLPQERC	N/A	U05781 (ZPB)	Nil
ZP3	Zona pellucida subunit 3	M20026	328–342	H-CSNSSSQFQIHGPR-OH	95%	AC004126 (ZP1) A18567	47% 31%

Amino terminus: H-free amine; carboxy terminus: -OH free acid, -NH₂ amide. Minimum purity was determined by reverse phase HPLC for all peptides except ZP1. The levels of peptide sequence identity are shown relative to the equivalent human sequences, where known, in the GenBank database.

^aThe mouse ZP1 peptide equivalent is deleted in the human ZP1 functional homolog (U05781; ZPB) but shows homology to sequences in a putative ZP1 gene described only by human genomic DNA analysis (AC004126; Hughes & Barratt 1999).

to BSA dissolved in PBS (1 mg/ml) at room temperature, to a final molar ratio of peptide to BSA of 40:1 (approximately 1 mg peptide/mg BSA). The solution was cooled to 4°C, and an equal volume of glutaraldehyde in water (2% v/v) was added, after which the solution was stirred at 4°C for 1 h. The glutaraldehyde was removed by dialysis against three changes of PBS, pH 7.4, and aliquots of BSA-conjugated peptide were stored at –20°C.

Assembly of polyepitope antigen DNA

The DNA constructs for polyepitope antigen A (AgA, GenBank Accession No. AY521451; Fig. 1A) and polyepitope antigen B (AgB, GenBank Accession No. AY521452; Fig. 1B) were as described (Hardy *et al.* 2002a). Two foreign immunodominant T helper epitopes, BRN-T from bovine ribonuclease (Chen *et al.* 1991) and HA-T from influenza virus hemagglutinin (Sadler *et al.* 1999), were included in both polyepitopes to enhance immune responses to the mouse-specific peptides.

The DNA construct for the ZP1 peptide (nucleotides 436–741, GenBank Accession No. U20448) fused to 6XHis (Fig. 1C) was produced by first cloning a 312 bp *SpeI/SalI* fragment (nucleotides 355–660) from AgB (Hardy *et al.* 2002a) into the *XbaI/SalI* sites of pMALcRI (New England Biolabs, Beverly, MA, USA) to produce pMAL:ZP1, followed by cloning a 377 bp *SacI/HindIII* fragment of pMAL:ZP1 into the *SacI/HindIII* sites of pQE31 (Qiagen) to produce pHis:ZP1.

The DNA construct for polyepitope antigen Z (AgZ, GenBank Accession No. AY521454; Fig. 1D) was assembled by sequential cloning of annealed oligomeric primers (Table 2) and PCR products. Specifically, a 372 bp PCR fragment containing peptide sequences for mouse ZP3 and ZP1 was produced from AgB (Hardy *et al.* 2002a) with primers mZP3aF and mZP1aR. Next, the 33 bp annealed linkers ZP3F2 and ZP3R2, and ZP3F3 and ZP3R3 were sequentially inserted into the *SpeI* site adjacent to the ZP3 sequence to produce AgZ (Fig. 1D). A 442 bp *XbaI/EcoRI* fragment containing the AgZ was then inserted into pMALcRI between the *XbaI* site and the *HindIII* site after it was end-filled, using Klenow enzyme, to produce pMAL:AgZ. AgZ contains three tandem repeats of a ZP3 contraceptive B-cell epitope (FQIHGPR) (Millar *et al.* 1989) and a T-cell epitope (NSSSSQFQ) (Rhim *et al.* 1992) upstream of the ZP1 peptide sequence. Although this T-cell epitope has previously been implicated in causing oophoritis (ovarian inflammation) in some strains of mice (Lou *et al.* 1995), it was included in the polyepitope antigens as it is mouse-specific and its importance for the induction of infertility is unclear (Bagavant *et al.* 1999, Lou & Borillo 2003). The synthetic primer sequences of the repeated ZP3 B-cell epitopes were modified relative to the published DNA sequence to prevent recombination between cloned sequences, but retain the same amino-acid translation.

A

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      BRN-T                      SP56
g s n c a y k t t q a n k l e c q a d s g l q n r l a l f t 30
ggatccAATTGTGCTTACAAGACGACACAGGCGAACAAActcgagTGTcAGGCAGACTCAGGCCTGCAAAACCGTCTTGCTCTTTTCACC 90

F P N I S E T N V T N K T Y L F G H E E N S T E H A M K G V 60
TTCCCAACATATCAGAAACCAATGTGACAAACAAACCTATCTATTGGTCATGAAGAAAACCAACCGAGCATGCCATGAAAGGTGTG 180
      Proliferin                      HA-T
C g t w f l q s d n e d a r i h s l y g m i s c s r a l n n 90
TGcggtagctGGTTCCTGCAATCTGACAATGAAGATGCTCGCATTTCATCTTTATATGGCATGATCAGCTGCTctagaGCGCTAAACAAC 270
      ZP3-T                      ZP3-B
R F Q I K G V E L K S r l n s s s s q f q i h g p r t s l k 120
CGGTTTCAGATCAAAGGTGTGAACTGAAAGCaagcttAATTCAAGCTCTTCACAGTTCAGATTTCATGGACCCCGCactagtCTGAAA 360
      Prolactin                      GMCSF
V P P E V L L N L I L S L V l e f v v s n e f s f k k l t c 150
GTCCCTCCGAAGTTCCTCTGAACCTGATCCTCAGTTTGGTGTggaattcGTCGTCTCTAACGAGTTCCTCTCAAGAAGCTAACATGT 450

v d 152
gtcgac 456

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B

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      BRN-T                      SP56
g s n c a y k t t q a n k l e c q a d s g l q n r l a l f t 30
ggatccAATTGTGCTTACAAGACGACACAGGCGAACAAActcgagTGTcAGGCAGACTCAGGCCTGCAAAACCGTCTTGCTCTTTTCACC 90

F P N I S E T N V T N K T Y L F G H E E N S T E H A M K G V 60
TTCCCAACATATCAGAAACCAATGTGACAAACAAACCTATCTATTGGTCATGAAGAAAACCAACCGAGCATGCCATGAAAGGTGTG 180
      Proliferin                      HA-T
C g t w f l q s d n e d a r i h s l y g m i s c s r a l n n 90
TGcggtagctGGTTCCTGCAATCTGACAATGAAGATGCTCGCATTTCATCTTTATATGGCATGATCAGCTGCTctagaGCGCTAAACAAC 270
      ZP3-T                      ZP3-B
R F Q I K G V E L K S r l n s s s s q f q i h g p r t s c p 120
CGGTTTCAGATCAAAGGTGTGAACTGAAAGCaagcttAATTCAAGCTCTTCACAGTTCAGATTTCATGGACCCCGCactagtTGTCCC 360

K P D H T V T P D P Y L A P P T T P E P F T P H A F A L H P 150
AAACAGACCACACCGTGACTCCGGACCCCTACCTGGCTCCACCCACCACCTGAGCCTTTTACACCTCATGCTTTTGCCCTTCACCCC 450

I P D H T L A G S G H T G L T T L Y P E Q S F I H P T P A P 180
ATCCCTGACCACACCTTGGCTGGGTCTGGCCACACTGGTCTCACTACGTTGTACCCAGAACAGAGCTTCATCCATCCAACCTCTGCTCCG 540

P S L G P G P A G S T V P H S Q W G T L E P W E L T E L D S 210
CCATCCCTAGGACCTGGACCTGCTGGATCCACTGACTCCCAATGGGGCACGCTGGAGCCCTGGGAATTGACTGAGCTGGATTCT 630

V G T H L P Q E R C v d 222
GTAGGTACCATCTGCCCCAGGAGCGGTGCGtcgac 666

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C

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      6XHis
m r g s h h h h h h t d p h a s s v p s s l v i e g r i s e 30
atgagaggatcgcatcaccatcaccatcacaggatccgcatcgagctcggtaccgtccctctctcgtgatcgagggaagatttcagaa 90
      ZP1
f g s s s c p k p d h t v t p d p y l a p p t t p e p f t p 60
ttcggatcctctagtTGTCCCAACACGACACACCGTGACTCCGACCCCTACCTGGCTCCACCCACCACACCTGAGCCTTTTACACCT 180

H A F A L H P I P D H T L A G S G H T G L T T L Y P E Q S F 90
CATGCTTTTGCCTTACCCCATCCCTGACCACACCTTGGCTGGGTCTGGCCACACTGGTCTCACTACGTTGTACCCAGAACAGAGCTTC 270

I H P T P A P P S L G P G P A G S T V P H S Q W G T L E P W 120
ATCCATCCAACCTCTGCTCCGCCATCCCTAGGACCTGGACCTGCTGGATCCACTGTACCTCACTCCCAATGGGGCACGCTGGAGCCCTGG 360

E L T E L D S V G T H L P Q E R C v d l q a s l i s * 146
GAATTGACTGAGCTGGATTCTGTAGGTACCCATCTGCCCCAGGAGCGGTGCGtcgacctgcaggcaagcttaattagctga 441

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D

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      ZP3-T                      ZP3-B (1)                      ZP3-B (2)
s r c s n s s s s s q f q i h g p r t s s q f q i h g p r q s s 30
tctagaTGTTCATAAGCTCTTCACAGTTCAGATTTCATGGACCCCGCactAGTCAATTCCAAATTCACGGGCCGAGGCAActctAGT 90
      ZP3-B (3)                      ZP1
Q F Q I H G P R Q s s c p k p d h t v t p d p y l a p p t t 60
CAATTTCAAATCCACGGTCTAGACAAtctagtTGTCCCAACACGACACACCGTGACTCCGACCCCTACCTGGCTCCACCCACCACA 180

P E P F T P H A F A L H P I P D H T L A G S G H T G L T T L 90
CCTGAGCCTTTTACACCTCATGCTTTTGCCCTTCACCCCATCCCTGACCACACCTTGGCTGGGTCTGGCCACACTGGTCTCACTACTTG 270

Y P E Q S F I H P T P A P P S L G P G P A G S T V P H S Q W 120
TACCAGAACAGAGCTTCATCCATCCAACCTCTGCTCCGCCATCCCTAGGACCTGGACCTGCTGGATCCACTGTACCTCACTCCCAATGG 360

G T L E P W E L T E L D S V G T H L P Q E R C * 143
GGCAGCTGGAGCCCTGGGAATTGACTGAGCTGGATTCTGTAGGTACCCATCTGCCCCAGGAGCGGTGCTAAccgcggtggagctc 446

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Figure 1 Nucleotide and deduced amino-acid sequences for polyepitope AgA (A), polyepitope AgB (B), ZP1 peptide fused to a 6XHis tag (C) and polyepitope AgZ (D). The locations of peptide sequences are shown above the sequences. The nucleotide and amino-acid sequences corresponding to the peptide sequences are shown in uppercase. The nucleotide and amino-acid sequences of the restriction sites introduced between the peptides during cloning steps and for bacterial expression are shown in lowercase.

Table 2 Oligonucleotides used to produce polypeptide AgZ. Nucleotide locations are relative to the sequence for AgZ (GenBank Accession No. AY521454).

Oligonucleotide	Sequence (5'–3')	Location
mZP3aF	TCTAGATGTTCTAATTCAAGCTCTTCAC	1–28
mZP1aR	CCGCGGTTAGCACCGCTCCTGGGGC	438–414
ZP3F2	CTAGTCAATTTCAAATCCACGGTCTAGACAAT	86–118
ZP3R2	CTAGATTGTCTAGGACCGTGGATTTGAAATTGA	122–90
ZP3F3	CTAGTCAATTCCAAATTCACGGGCCGAGGCAAT	53–85
ZP3R3	CTAGATTGCCTCGGCCCGTGAATTTGGAATTGA	89–57

Protein purification

Recombinant 6XHis fused ZP1 peptide was prepared and affinity purified with Ni-NTA Superflow resin (Qiagen) in 6 M urea and renatured by sequential dialysis according to the manufacturer's recommendations. Recombinant AgZ fused to MBP was prepared and affinity purified with amylose resin as previously described (Hardy & Holland 1996). All recombinant protein expression was conducted with *E. coli* strain BL21 CodonPlus-RIL (Stratagene, La Jolla, CA, USA). Polyacrylamide gel electrophoresis was conducted by standard procedures (Coligan *et al.* 1995), with low-range prestained proteins (BioRad, Hercules, CA, USA) as molecular weight markers. Molecular weight predictions were conducted with the Swiss-Prot analysis tool (Boeckmann *et al.* 2003) on the ExPASy server (Gasteiger *et al.* 2003).

Immunization and mating protocols

The Animal Experimentation and Ethics Committee of the Commonwealth Scientific and Industrial Research Organization, Division of Sustainable Ecosystems, approved all experimental procedures involving the use of mice, in compliance with the Australian National Health and Medical Research Council guidelines.

BALB/c mice (6–8 weeks old) were obtained from the Animal Resources Centre, Murdoch, Perth, Australia. CBA mice (6–8 weeks old) were obtained from the Australian National University Animal Resources Centre, Canberra, Australia. Female mice were immunized by intraperitoneal (i.p.) injection with 20 µg peptide conjugate or recombinant protein in 100 µl 50% PBS and 50% Freund's complete adjuvant (FCA) (Sigma), and boosted by i.p. injection three or four times with 20 µg peptide conjugate in 100 µl 50% PBS and 50% Freund's incomplete adjuvant (FIA) (Sigma) at 2-week intervals. Blood samples were collected before each boost and 2 weeks after the final boost from the suborbital sinus. The blood was then spun at 1500 r.p.m. for 5 min, and the sera were collected and stored at –20 °C until tested.

Female BALB/c mice were coupled 2 weeks after the final immunization with proven fertile BALB/c males of similar age. Males were removed after 2 weeks and the females allowed to litter. Female CBA mice were coupled 2 weeks after the final immunization with CBA males, and the males were removed after 2 weeks and the females

allowed to litter. Fresh males were then introduced to any female CBA mice that failed to litter (5 weeks after the final immunization) for a further 2 weeks, and the females again allowed to litter. After littering was completed, pups were killed and the females were autopsied.

Histology

Female mice were killed by cervical dislocation and the ovaries were collected and fixed in Bouin's fixative (71.4% saturated picric acid, 9.5% formaldehyde and 4.8% glacial acetic acid). Bouin's fixative was replaced by 70% ethanol before tissues were embedded in paraffin wax. Tissue sections (7 µm) were stained with hematoxylin and eosin.

Assay of antibodies

Serum IgG antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) in 96-well microtiter plates (Greiner, Labortechnik GmbH, Frickenhausen, Germany, or Nalge Nunc, Rochester, NY, USA). Sera from mice recognized unconjugated peptides for all peptides except GMCSF and HSP when they were used as detection antigens in ELISA. Peptides GMCSF and HSP were therefore conjugated to BSA to improve detection. Unconjugated peptides or peptides conjugated to BSA were suspended in binding buffer (50 mM NaHCO₃ pH 9.6) and added to microtiter plates (1.0 µg antigen in 50 µl/well) and incubated for either 1 h at 37 °C or overnight at 4 °C. After the incubation, wells were washed three times with ELISA buffer (145 mM NaCl, 7.5 mM Na₂HPO₄ and 2.5 mM NaH₂PO₄, containing 0.005% Tween-20, pH 7.4). Nonspecific 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 serum samples 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 affinity-purified and horseradish peroxidase-labeled goat anti-mouse IgG (Silenus Laboratories, Hawthorn, Australia) 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 color developed by adding 50 µl development buffer. Development buffer consisted of 0.01% H₂O₂,

Figure 2 SDS–PAGE gel stained with Coomassie brilliant blue showing purified recombinant peptide antigens. Lane 1, polyepitope AgZ fused to maltose-binding protein; lane 3, ZP1 peptide fused to 6XHis tag; lane 2, molecular mass markers.

2 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma), in acetate buffer (100 mM sodium acetate and 50 mM NaH₂PO₄, pH 4.0) and was prepared immediately prior to use. Reactions were terminated after 20 min at 20 °C by addition of 50 µl 10% SDS, and absorbance was measured at 405 nm on an ELISA plate reader (Bio-Rad). Means of duplicate samples are reported.

Table 3 Fertility of mice immunized with different peptide antigen formulations. Female BALB/c or CBA mice were immunized and boosted 3–4 times at 2-week intervals with antigen in Freund's adjuvants. Individual or groups of peptides were either keyhole limpet hemocyanin (KLH) conjugates or fused to a 6XHis tag (peptide ZP1). Recombinant polyepitopes (AgA, AgB and AgZ) were fused to maltose-binding protein (MBP). BALB/c mice were mated 2 weeks after the final boost. CBA mice were mated 2 and 5 weeks after the final boost. Significant differences ($P < 0.05$) from controls (PBS or KLH) are shown in bold type.

Mouse strain	Peptide antigen	Total mice	Total boosts	Fertile mice	Fertility (%)	Total pups	Litter size of fertile mice (mean ± S.E.M.)	P^a
BALB/c	SP56	5	3	2	40	6	3.0 ± 1.3	0.03
	GMCSF	5	3	2	40	7	3.5 ± 1.4	0.05
	PRL	5	3	2	40	12	6.0 ± 1.7	0.60
	SP56 + GMCSF + PRL	6	3	3	50	16	5.3 ± 1.1	0.22
	LIFR	5	3	3	60	13	4.3 ± 1.2	0.09
	PLF	5	3	3	60	17	5.7 ± 1.1	0.34
	HSP	5	3	4	80	20	5.0 ± 1.0	0.13
	OGP	5	4	5	100	32	6.4 ± 0.8	0.55
	ZP1	10	3	9	90	47	5.2 ± 0.7	0.08
	AgA ^b	10	3	6	60	33	5.5 ± 0.9	0.24
	AgB ^b	10	3	4	40	21	5.3 ± 1.1	0.23
	AgZ	9	3	7	78	24	3.4 ± 0.9	0.01
	MBP ^b	10	3	7	70	52	7.4 ± 0.8	0.71
	PBS	10	3	8	80	56	7.0 ± 0.8	
	ZP3 ^c	10	4	9	90	32	3.6 ± 0.6	0.07
CBA	KLH ^c	14	4	11	79	55	5.0 ± 0.5	
	AgZ	6	3	3	50	16	5.3 ± 1.1	0.93
	PBS	6	3	5	83	26	5.2 ± 0.9	

^a Probability that the experimental treatment was significantly less than control values.
^b Data from Hardy *et al.* (2002a).
^c Data from Hardy *et al.* (2002b).

Statistical analyses

Results are presented as means ± S.E.M. Data were analyzed by two-tailed Student's *t*-test (GenStat for Windows 2002, 6th edn, VSN International Ltd, Hemel Hempstead, UK). A value of $P < 0.05$ was considered significant.

Results

Purification of recombinant proteins

The predicted sizes and molecular masses MBP:AgZ and 6XHis:ZP1 were 530 amino acids and 58 kDa, and 146 amino acids and 16 kDa respectively. The purified 6XHis:ZP1 peptide appeared as a single 20 kDa band on SDS–PAGE gels (Fig. 2, lane 3), slightly higher than the predicted molecular mass of 16 kDa for the 146-amino-acid product. The purified MBP:AgZ appeared as two main products of 48 and 50 kDa, slightly lower than the predicted molecular mass of 58 kDa for the 530-amino-acid fusion protein (Fig. 2, lane 1). A minor, higher molecular weight product of 70 kDa was also present in the MBP:AgZ preparation.

Fertility of mice immunized with peptide antigens

The most effective peptides for reducing fertility in mice were SP56, GMCSF and PRL, and only 40% of mice in each of these groups produced litters compared with PBS controls where 80% of mice were fertile (Table 3). The use of these three peptides in combination also reduced

fertility in mice, but was slightly less effective (50% of mice produced litters) than when peptides were used individually. Mice immunized with two of the peptides (SP56 and GMCSF) chemically coupled to KLH produced significantly fewer pups than controls ($P=0.03$ and 0.05 respectively), indicating that the effect of these two peptides was to cause a reduction in the mean litter sizes of mice (Table 3). Peptides LIFR and PLF also appeared to reduce fertility, although to a lesser extent (60% of mice produced litters). The remaining peptides (HSP, OGP and ZP3 conjugated to KLH, or ZP1 fused to a 6XHis tag) did not significantly affect the fertility of female BALB/c mice.

Fertility of mice immunized with polyepitope antigens

Fertility of BALB/c mice immunized with AgA (60%) and AgB (40%) has been previously reported (Hardy *et al.* 2002a). Fertility of mice immunized with AgZ (78%) was similar to the control groups immunized with PBS (80%) or MBP (70%). Although most BALB/c mice produced litters after immunization with AgZ, there was a highly significant reduction ($P=0.01$) in the mean litter sizes of the mice that produced litters compared with PBS-treated controls (Table 3).

In a separate experiment, we assessed the fertility of CBA mice immunized with MBP:AgZ under identical conditions to BALB/c mice. However, in this experiment, the CBA mice had generally low fertility and delayed breeding compared with the BALB/c mice. After the mice were first mated 2 weeks after the final boost, only 2/6 AgZ-treated and 2/6 PBS-treated mice had produced litters. One further AgZ and three PBS-treated mice produced litters when fresh males were introduced 5 weeks after the final boost to the mice that failed to litter in the first mating. Overall, by 8 weeks after the final boost, the proportion of CBA mice that produced litters after immunization with AgZ (50%) was less than PBS controls (83%), although no significant difference in mean litter sizes was present between the fertile mice in the two groups (Table 3).

Antibody responses in mice immunized with peptide antigens

Most female BALB/c mice immunized by repeated i.p. injection with conjugated peptides produced detectable peptide-specific IgG antibodies by 2 weeks after the second boost (Fig. 3), and these subsequently increased in many but not all mice after a further boost.

Mice immunized with individual peptides developed consistently strong antibody responses against LIFR, ZP1 and ZP3. In contrast, peptide-specific IgG antibodies against the remaining peptides were either highly variable (GMCSF, PLF, PRL, OGP and SP56) between individual mice or absent (HSP). In particular, significant IgG responses (absorbance of >0.1 at 1:100 serum dilution) were produced after immunization in only a fraction of individuals for peptides PLF (2/5 responders) and OGP (2/5).

Mice immunized with a combination of three peptides (SP56, GMCSF and PRL) had similar response profiles to each peptide as mice immunized with the single peptides. However, individual mice immunized with single peptides developed considerably higher peptide specific antibodies than mice immunized with the same peptide in combination with others, with levels up to 2-fold higher for GMCSF and PRL and as much as 10-fold higher for SP56 (Fig. 3).

Antibody responses in mice immunized with polyepitope antigens

Previously, mice immunized with AgA and AgB were shown to produce strong antibody responses to the polyepitopes, but the response to the component peptides was not assessed (Hardy *et al.* 2002a). Sera from these mice collected 2 weeks after the final boost were therefore tested for the presence of peptide-specific antibodies, and only certain peptides were recognized in ELISA. All 10 mice immunized with AgA (containing GMCSF, PLF, PRL, SP56 and ZP3 peptide sequences) produced SP56-specific antibodies, two had traces of ZP3 antibodies (absorbance = 0.13, 0.16) and none developed GMCSF, PLF or PRL-specific antibodies (Fig. 4). All 10 mice immunized with AgB (containing PLF, SP56, ZP1 and ZP3 peptide sequences) produced SP56-specific and ZP1-specific antibodies (Fig. 4), but failed to produce antibodies against PLF or ZP3.

The absence of significant ZP3 antibody responses in mice immunized with AgA and AgB led us to produce a third polyepitope (AgZ). This contained the immunodominant peptide from AgB (ZP1) and three tandem repeats of the ZP3 peptide containing a known contraceptive B-cell epitope (Fig. 1D). This was to test whether the tandem repeats were able to enhance the production of ZP3-specific antibodies while not affecting responses to ZP1. All 10 female BALB/c mice immunized with AgZ produced both ZP1 and ZP3 antibodies (Fig. 4) at levels comparable to mice immunized with the individual peptides. The ability to induce ZP3 antibodies in the mice, however, is dependent on the mouse strain. CBA mice, reportedly resistant to the immunocontraceptive effects of ZP3 antigens (Lloyd *et al.* 2003), were immunized with AgZ under identical conditions to BALB/c mice but generated antibody responses to only the ZP1 peptide (Fig. 4).

Relationship between antibody responses and fertility in mice immunized with peptide and polyepitope antigens

The relative levels of peptide-specific antibody responses of fertile and infertile mice immunized with conjugated peptides were compared with those induced by the same sequences delivered as recombinant polyepitopes. The final antibody response to any given peptide by individual mice was not indicative of whether the animals were subsequently found to be infertile (Figs 3 and 4).

Figure 3 Progression of serum IgG antibody responses by ELISA in female BALB/c mice immunized and boosted three times at 2-week intervals with peptides in Freund's adjuvants. Individual or groups of peptides were either keyhole limpet hemocyanin (KLH) conjugates or fused to a 6XHis tag (peptide ZP1). SP56 (S + G + P), GMCSF (S + G + P) and PRL (S + G + P) indicate response to SP56, GMCSF and PRL respectively from mice immunized with mixed peptides SP56, GMCSF and PRL. Absorbance values are shown for sera diluted 1:100 (SP56 was diluted 1:1000). Open symbols show individual mice that were subsequently infertile upon mating at 8–10 weeks.

Figure 4 Peptide-specific serum IgG antibody responses in mice immunized with recombinant polyepitope antigens. Female BALB/c or CBA mice were immunized in Freund's adjuvants and boosted three times at 2-week intervals with AgA, AgB or AgZ fused to maltose-binding protein. Values are mean $\pm$ S.E.M. absorbance values at 405 nm (1:100 serum dilutions) by ELISA against the peptides for each mouse, 2 weeks after the final immunization. Open bars indicate infertile mice; hatched bars indicate mice with 1–3 pups; solid bars indicate mice with more than three pups.

Nevertheless, significant differences were present between the levels of peptide-specific antibodies produced by fertile and infertile mice immunized with two of the antigen formulations. Specifically, peptide-specific antibody levels were significantly higher for peptide SP56 in infertile compared with fertile BALB/c mice immunized with AgB (absorbance = 1.5 ± 0.1 vs 1.1 ± 0.1 respectively; $P = 0.05$) and for peptide ZP3 in infertile compared with fertile BALB/c mice immunized with AgZ (absorbance = 2.7 ± 0.6 vs 0.6 ± 0.3 respectively; $P = 0.015$).

Ovarian effects in mice treated with polyepitope antigen Z

Ovaries of CBA and BALB/c mice immunized with AgZ and ZP1 were examined for evidence of ovarian pathology 8 weeks after the final immunization, once production of litters had been completed (Fig. 5). Ovaries from all mice immunized with PBS contained primary, preantral and antral follicles, and corpora lutea were also present in mice that produced litters (Fig. 5A and B). Likewise, ovaries from all BALB/c mice and fertile CBA mice immunized with AgZ (Fig. 5C) contained primary, preantral and antral follicles as well as corpora lutea. However, ovaries from infertile CBA mice immunized with AgZ

were abnormal, lacked antral follicles and contained very few primary or preantral follicles (Fig. 5D). Ovaries from all 6XHis:ZP1-treated BALB/c mice, including the single infertile animal, appeared normal and contained all stages of follicles and corpora lutea (Fig. 5E and F).

Discussion

We have demonstrated that fertility of mice is significantly reduced by up to 50% after immunization with peptide and polyepitope antigens that target a range of proteins involved in reproduction. All peptides were selected on the basis of low amino-acid sequence identity to the corresponding sequences from homologous proteins in other species and are therefore considered to represent potential mouse-specific contraceptive antigens. Peptides were produced by two independent methods: either chemically synthesized and conjugated to a carrier protein or expressed in bacteria as recombinant polyepitopes.

Mice were immunized with conjugated peptides using protocols that have previously been shown to induce both strong peptide-specific antibody responses and infertility (Millar *et al.* 1989, Sun *et al.* 1999, Hardy *et al.* 2002b). Most mice developed peptide-specific antibodies in

Figure 5 Ovarian sections of mice immunized with polyepitope AgZ or ZP1 peptide. Female BALB/c or CBA mice were immunized in Freund's adjuvants and boosted three times at 2-week intervals with AgZ fused to maltose-binding protein or ZP1 fused to a 6XHis tag. Fertility of mice was determined by mating 3 weeks prior to collection of ovaries. Ovaries are from PBS-treated fertile (A) and infertile (B) or AgZ-treated fertile (C) and infertile (D) CBA mice, 8 weeks after the final boost, or from ZP1-treated fertile (E) or infertile (F) BALB/c mice, 5 weeks after the final boost. CL, corpora luteum. Solid arrows indicate antral follicles; open arrows indicate preantral follicles.

response to immunization with conjugated peptides, although there was considerable variation in the levels between individual mice. Peptide-specific IgG antibody levels in mice immunized with SP56 + GMCSF + PRL were 2–10-fold less than in mice immunized with single peptides, indicating that suppression of epitope-specific antibodies occurred when mice were immunized with multiple peptides in conjugated form. The most consistent antibody responses were to the two peptides derived from zona pellucida proteins (ZP1 and ZP3). As the ZP1 and ZP3 proteins are not expressed until follicles commence development around birth (Millar *et al.* 1993, Epifano *et al.* 1995), self-reactive T helper cells may not be deleted for zona pellucida peptides as for other self-proteins (MacLennan 1995), enabling more consistent induction of antibody responses. Nevertheless, there was no absolute link between antibody levels and infertility with any of the peptide antigens, and this is comparable to results reported elsewhere (Lea *et al.* 1998). This suggests that other factors, such as T-cell responses or antibody avidity and isotype, in combination with antibody levels, are responsible for the immunocontraceptive effects we observed.

Polyepitope antigens (AgA and AgB) containing mouse-specific peptides have previously been shown to reduce fertility in mice (Hardy *et al.* 2002a). In the present study, we have shown that the antibody responses were preferentially directed against epitopes in the longer peptide sequences. These longer peptides (SP56 and ZP1) are more likely to contain multiple antigen-specific T- and B-cell epitopes, and this could explain the production of antibodies preferentially against them in the polyepitopes. This result demonstrates the potential disadvantage of adopting a peptide approach to contraceptive vaccine design, since immunodominance of epitopes can occur by selective and dominant activation of peptide-specific B cells (Ju *et al.* 1993).

Previously it has been shown that BALB/c mice immunized with synthetic peptides containing multiple ZP3 epitopes elicit higher ZP3-specific antibodies and show greater infertility than those immunized with single peptides (Sadler *et al.* 1999). Therefore, a polyepitope antigen (AgZ) was produced that contained three tandem repeats of a ZP3 B-cell epitope to test whether repeated epitopes could overcome the observed lack of antibody responses to ZP3 in mice immunized with AgA and AgB. The ZP1 peptide was included in AgZ as it was previously reported to have contraceptive effect (Hardy *et al.* 2002a) and consistently induced antibodies. Indeed, BALB/c mice immunized with AgZ produced both ZP1 and ZP3 antibodies, although there was considerable variation in the ZP3 antibody levels (over 100-fold difference) between individual mice. Comparable variation (30-fold differences between individual mice) was reported in mice after immunization with the synthetic ZP3 peptides (Sadler *et al.* 1999). Importantly, BALB/c mice immunized with AgZ and AgB produced similar levels of ZP1 antibodies, demonstrating

that recombinant proteins encoding repeated epitopes are able to elicit antibody responses while not affecting responses to adjacent peptides. In contrast, CBA mice immunized with AgZ showed lower levels of ZP1-specific antibodies than BALB/c mice, and did not produce detectable ZP3-specific antibodies. Levels of antibodies induced in response to immunization with repeated ZP3 B-cell epitopes are therefore dependent on mouse strain, as has been reported elsewhere for polyepitopes comprising multiple peptides (Chai *et al.* 1992).

Although the proportion of BALB/c mice immunized with AgZ that produced litters (78%) was not significantly different from controls (80%), there was a highly significant reduction in the mean litter sizes in the fertile mice, and this correlated with the levels of ZP3-specific antibodies. The only two infertile mice had the highest antibody levels, and two mice with intermediate antibody levels each had only one pup. There was no evidence of pathologic effects on the ovaries of BALB/c mice immunized with AgZ, despite the inclusion of a ZP3 T-cell epitope (NSSSSQFQ) that induces oophoritis (ovarian inflammation) in some mouse strains (Rhim *et al.* 1992). The interference in fertility in BALB/c mice is therefore probably due to prevention of fertilization or implantation by ZP3-specific antibodies. By contrast, 50% of CBA mice immunized with AgZ lacked developing follicles and were infertile without any evidence for the presence of ZP3-specific antibodies. This rate of ovarian pathology is comparable to the reported incidence of oophoritis in CBA mice exposed to the NSSSSQFQ epitope (Lou *et al.* 1995), although mice that develop oophoritis after passive immunization with ZP3 peptide-induced T cells are fertile (Bagavant *et al.* 1999). Our results do, however, suggest that an alternative to antibody-mediated infertility occurs in CBA mice, perhaps through T cell-mediated destruction of developing follicles.

The apparent association of ZP1 and ZP3 antibodies with contraceptive effects in some mice immunized with recombinant polyepitopes, but not with the conjugated peptides, may reflect the manner in which antigens were prepared. Polyepitopes were expressed as soluble proteins in *E. coli*, and this may have provided conformational B-cell epitopes that were absent from the peptides. Alternatively, novel epitopes for the ZP1 or the adjacent ZP3 peptides may have been created at junction sites in the polyepitopes, as has been reported in other studies (Dalum *et al.* 1996, Partidos & Kanse 1997). However, we did not test whether such novel or conformational epitopes for ZP1 or ZP3 were created in the polyepitopes in the present study.

On the basis of our results, we suggest that improvements to species-specific contraceptive peptide vaccines can be addressed in several ways. Firstly, contraceptive antibody responses to immunization with polyepitopes could be improved through the use of repeated epitopes. Increasing copy number has been shown to correlate with corresponding increases in contraceptive antibody levels

both by our studies and by others. The number (Marussig *et al.* 1997, Oscherwitz *et al.* 1999), orientation (Partidos *et al.* 1992) and use of immune enhancers such as cytokines (Ramsay & Ramshaw 1997) can be applied to improve the immunogenicity of subdominant epitopes. Secondly, the main criteria for selecting peptides in this study were to test combinations of peptides from key proteins for a broad a range of physiological targets. Polyepitope vaccines that simultaneously target species-specific regions of multiple proteins with similar structural or functional importance, such as all three zona pellucida proteins ZP1, 2 and 3, might induce more effective contraception. This approach is supported by our demonstration that contraceptive effectiveness is increased when the ZP1 and ZP3 are used in combination and that contraceptive mouse-specific ZP2 peptides are likely to exist (Hinsch *et al.* 1998, Sun *et al.* 1999). Thirdly, nonantibody immune responses, such as cytotoxic T-cell lymphocytes (CTLs), may generate immunocontraceptive effects and contraception after immunization with the ZP3 peptide has been reported to require both T-cell and antibody responses (Lou & Borillo 2003). The identification of T-cell epitopes able to induce CTLs for appropriate reproductive targets and their incorporation into polyepitopes is likely therefore to improve immunogenicity, in a similar manner to those included in anticancer polyepitope vaccines now under development (Suhrieb 2002). Finally, replicating virus vaccines expressing the entire mouse ZP3 protein (Jackson *et al.* 1998, Lloyd *et al.* 2003) appear considerably more effective at inducing infertility than when ZP3 protein alone is used to immunize mice (Hardy *et al.* 2003). Therefore, delivery of polyepitope antigens using recombinant virus delivery systems could be expected to improve contraceptive effects.

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