Assessment of contraceptive vaccines based on recombinant mouse sperm protein PH20

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Abstract

Mouse PH20 (mPH20), the mouse homologue to guinea pig hyaluronidase protein PH20 (gpPH20), was used to produce contraceptive vaccines that target both sexes of mice. Previously, immunization with a female gamete antigen (the zona pellucida subunit 3 protein) delivered in a recombinant murine cytomegalovirus (MCMV), or as a purified recombinant protein, has been shown to induce infertility in female mice. There is evidence, however, that sperm protein antigens could provide broader contraceptive coverage by affecting both males and females, and the most promising has been gpPH20 when tested in a guinea pig model. Mice were therefore either inoculated with a recombinant MCMV expressing mPH20 or immunized directly with purified recombinant mPH20 protein fused to maltose-binding protein. Mice treated with either vaccine formulation developed serum antibodies that cross-reacted to a protein band of 55 kDa corresponding to mPH20 in Western blots of mouse sperm. However, there was no significant reduction in the fertility of males or females compared with control animals with either formulation. We conclude from our data that recombinant mPH20 is not a useful antigen for inclusion in immunocontraceptive vaccines that target mice.

Reproduction (2004) **127** 325–334

Introduction

Mouse PH20 (mPH20), also termed murine sperm adhesion molecule 1 (SPAM-1), is a protein that is present in the male reproductive tract. It is expressed in both the testis and epididymis and is present in sperm and in the luminal fluid of the epididymis (Deng *et al.* 1999, 2000, Zhang & Martin-DeLeon 2003*a*). The PH20 protein is widely conserved among mammals, and homologues have been cloned from many species including guinea pig, where the protein was first identified (Lathrop *et al.* 1990), rat (Hou *et al.* 1996), rabbit (Holland *et al.* 1997), fox (ten Have *et al.* 1998), pig (Day *et al.* 2002), monkey and man (Lin *et al.* 1993).

PH20 exists as a membrane-bound protein on the surface of sperm (Li *et al.* 1997) and appears to have several roles in fertilization in various species (Hunnicutt *et al.* 1996, Cherr *et al.* 2001, Vines *et al.* 2001). First, PH20 possesses hyaluronidase activity that facilitates transfer of the sperm through the cumulus layer surrounding the unfertilized egg (Gmachl *et al.* 1993, Lin *et al.* 1994). Secondly, PH20 is able to bind directly to the zona pellucida during the early steps of fertilization (Primakoff *et al.* 1985). This is thought to be due to the ability of PH20 to bind hyaluronic acid (HA) in the zona pellucida, a process that is independent of its hyaluronidase activity (Hunnicutt *et al.* 1996, Yudin *et al.* 1999). Finally, the binding of PH20 to HA indirectly triggers an increase in the intracellular calcium of sperm that leads to an increase in the responsiveness of sperm to induction of the acrosome reaction (Cherr *et al.* 2001).

Immunization with affinity purified PH20 from guinea pig sperm (gpPH20) has been shown to cause complete and reversible infertility in both female and male guinea pigs after a single dose of antigen (Primakoff *et al.* 1988, 1997, Tung *et al.* 1997). In males, immunization with gpPH20 in Freund's adjuvant led to complete infertility due to the loss of normal sperm in the epididymis and associated autoimmune orchitis (Tung *et al.* 1997), whereas it has been proposed that infertility in females is

by prevention of sperm–egg binding by PH20 antibodies (Primakoff *et al.* 1988).

In order to produce contraceptive vaccines based on sperm that are suitable for vaccinating large numbers of individuals, it is necessary to use recombinant forms of the proteins to generate sufficient quantities of the antigens. However, recombinant sperm proteins are generally less effective than the purified native sperm proteins in inducing an immunocontraceptive response (O'Rand & Lea 1997, Naz 1999). In addition, the use of infectious sterilizing agents would avoid the need for direct inoculation and would provide a means for controlling widespread populations of pest animal species through natural transmission of the vaccines (Tyndale-Biscoe 1994).

Recombinant viruses provide one means of delivery of contraceptive antigens and are effective in reducing the fertility of mice (Jackson *et al.* 1998, Lloyd *et al.* 2003). These vaccines have employed female gamete antigens and affect the fertility of female mice only. However, the use of a sperm protein such as PH20 has considerable potential as a recombinant vaccine antigen because it can induce an immunocontraceptive response in male and female animals. This concept has yet to be tested in mice.

The virus vector that has been chosen for the development of immunocontraceptive vaccines for biological control of wild mice in Australia is murine cytomegalovirus (MCMV). This beta-herpesvirus has been selected because it is highly species-specific, it is present in all populations of Australian mice tested to date and multiple strains of it are able to infect a single host animal, indicating that pre-immunity to the virus may not interfere with delivery of a recombinant virus in the field (Shellam 1994, Lloyd *et al.* 2003). Therefore, in order to test the vaccine potential of PH20 in mice, we have cloned and characterized mPH20 from BALB/c mice and constructed a recombinant MCMV expressing this protein. The effect of this vaccine on fertility has been tested in male and female mice.

Materials and Methods

Oligonucleotides

Oligonucleotides for cDNA synthesis and PCR were synthesized by the Biomolecular Resources Facility (ANU, Canberra, ACT, Australia). Oligonucleotide pairs required for direct annealing to produce double-strand linker DNAs were phosphorylated at the 5′-ends to enable direct cloning into DNA plasmids after annealing without further manipulation, and were synthesized by Life Technologies (Mt Waverly, Victoria, Australia). The cDNA synthesis primer for reverse transcription 5′-GCAAAAATAATAATAATAAT-3′ was designed against the 3′-untranslated region of the mPH20 sequence (GenBank accession number U33958, nucleotides 1897–1878). Oligonucleotide primers for PCR were designed to amplify 1563 bp of cDNA containing the entire coding region of mPH20. Primers were PH20FOR

5′-gtggaattcGTGATGGGAGAGTTGAGA-3′ (nucleotides 310–327, including the ATG start codon at 313–315), and PH20REV 5′-ggagaattcAAACGCTGTGGGTAGAG-3′ (nucleotides 1872–1856, 5 nucleotides downstream of the TAG stop signal). The primers included EcoRI restriction enzyme sites (nucleotides shown in lower case) to facilitate cloning of the PCR product.

PCR cloning of mPH20

Polyadenylated RNA was isolated from the testes of inbred BALB/c mice, as previously described (Chomczynski & Sacchi 1987), and reverse transcribed into cDNA using a TimeSaver cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). PCR was conducted in an FTS-320 thermal sequencer (Corbett Research, Lidcombe, Sydney, Australia). The resulting 1581 bp PCR product was subcloned into pBluescriptII KS− to produce plasmid pCMH155 in *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA). The authenticity of the clone was confirmed by sequencing the entire insert using dye-labelled terminators on a Perkin-Elmer ABI automated sequencer (Biomolecular Resources Facility, ANU, Canberra, ACT, Australia). DNA sequences were analysed using Geneworks 2.3 (Intelligenetics Inc., Mountain View, CA, USA) and Vector NTI (InforMax, North Bethesda, MD, USA) software. Sequence comparisons were made using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) and Entrez software (Schuler *et al.* 1996).

Expression and purification of recombinant mPH20

The mPH20 cDNA coding region, minus the first 29 amino acids from the amino terminus that encode a secretory signal sequence to help increase protein yields, was subcloned into the bacterial expression systems pMAL (New England Biolabs, Beverly, MA, USA) and pGEX (Pharmacia Biotech, Uppsala, Sweden). Specifically, an 861 bp HinfI/XbaI DNA fragment containing the 5′ region of the mPH20 cDNA of pCMH155 was purified on a 1% agarose gel. The HinfI site was end-filled using Klenow enzyme prior to restriction with XbaI to produce a blunt-ended HinfI/XbaI fragment. Next, a 640 bp XbaI/SalI DNA fragment of pCMH155, containing the 3′ region of the mPH20 cDNA, was purified on a 1% agarose gel. The two fragments were then inserted into the bacterial expression vector pMal-c cut with StuI and SalI in a three-way ligation to produce pCMH169 where the 5′ truncated mPH20 coding region was fused in frame to the maltose-binding protein (MBP) sequence. A 1535 bp EcoIcRI/SalI DNA fragment of pCMH169 was subsequently subcloned into pGEX-4T-1 digested with SmaI and SalI to produce pCMH309, where the truncated mPH20 coding region was fused in-frame with the glutathione-*S*-transferase (GST) sequence. MBP for the control experiments was prepared using the vector pMAL-cR1dH (Hardy *et al.* 2002*a*).

Bacterial cell extracts containing recombinant MBP fused to mPH20 (MBP-mPH20), MBP, GST fused to

mPH20 (GST-mPH20) and GST proteins were prepared in *E. coli* strains BL21-CodonPlus-RIL or XL-1 Blue (Stratagene, La Jolla, CA, USA), as previously described (Hardy & Holland 1996). MBP proteins were then affinity purified using amylose resin, as previously described (Hardy & Holland 1996). GST proteins were affinity purified using glutathione sepharose 4B resin (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's recommendations.

Construction of MCMV expression plasmid vectors

The entire mPH20 coding sequence was cloned into a mammalian gene expression plasmid vector (pCMH149, GenBank accession number AY122060) derived from pCMV-beta (Clontech, GenBank accession number U02451). The plasmid pCMH149 contains a multiple cloning site (MCS) for insertion of genes under the control of the human cytomegalovirus (hCMV) *ie1* gene promoter, an intron to enhance gene expression, and a simian virus 40 gene terminator. The pCMH149 plasmid was produced as follows. The β-galactosidase gene of plasmid pCMV-beta was excised using NotI and replaced with a multiple cloning site linker, and the unique SalI and EcoRI sites bounding the expression cassette were replaced with NotI linkers. Plasmid pCMH155 was restriction enzyme digested with HindIII, end-filled using Klenow enzyme and re-ligated to produce pCMH156 with the HindIII site removed. A 1612 bp XmaI/SalI fragment of pCMH156 was then cloned into the XmaI/SalI of pCMH149 to produce the mPH20 expression plasmid pCMH157.

Construction of MCMV transfer plasmid vectors

The MCMV transfer plasmid vector pH3L contains the entire 7161 bp HindIII L fragment of MCMV strain K181 inserted into plasmid pGEM11 (Lloyd *et al.* 2003). The 79 bp HpaI fragment of pH3L within the MCMV *ie2* gene was removed and replaced by a NotI linker to produce pH3LN. Next, the 2672 bp NotI fragment of pCMH157 containing the mPH20 expression cassette was inserted into pH3LN (ensuring that mPH20 gene expression was in the same direction as the MCMV *ie2* gene) to produce the MCMV-mPH20 transfer plasmid, pCMH158.

Generation of recombinant (r) MCMV-mPH20

The rMCMV strain K181 containing the mPH20 expression cassette (rMCMV-mPH20) was produced by homologous recombination, as previously described (Lloyd *et al.* 2003). Briefly, semi-confluent primary mouse embryo fibroblasts (MEF) in six-well trays were co-transfected with 10–50 μg of infectious DNA (MCMV strain K181, substrain RM427 + , kindly provided by Prof. E. Mocarski, Stanford University, Stanford, CA, USA) and 2 μg pCMH158 linearized with HindIII restriction enzyme. Cells were transfected by a calcium phosphate method using the CellPhect kit (Amersham Pharmacia

Biotechnology UK Ltd, Buckinghamshire, UK) according to the manufacturer's instructions. At the completion of the incubation period, the cells were osmotically shocked with 1% glycerol in buffered saline with the osmolarity of mouse serum (MOBS, 6.5 mmol Na_2HPO_4/l, 1.5 mmol KH_2PO_4/l, 2.7 mmol KCl/l and 16.8 mmol NaCl/l, pH 7.3) for 1 min. Viruses with the mPH20 expression cassette inserted by homologous recombination were detected as white plaques after addition of X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) due to replacement of a bacterial β-galactosidase expression cassette inserted in the *ie2* gene of RM427 + . White plaques were picked and plaque was purified three times before generation of a high titre stock of rMCMV-mPH20. The correct insertion of the mPH20 expression cassette into MCMV was confirmed by Southern blot analysis. Viral DNA was prepared from rMCMV-mPH20, as previously described (Lloyd *et al.* 2003), and restriction fragment length polymorphism was performed using the restriction enzymes HindIII and EcoRV. Fragments were separated on a 0.9% agarose gel and Southern blots performed using DNA fragments labelled with ^{32}P-dCTP (Megaprime DNA labelling kit, Amersham) and standard protocols (Sambrook *et al.* 1989).

Immunization and mating protocols with recombinant proteins

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 National Health and Medical Research Council of Australia (NHMRC) guidelines. BALB/c mice were obtained from the Animal Resources Centre (Murdoch, Perth, Australia). An i.p. delivery route using Freund's adjuvants was used to generate mPH20-specific antibodies and to test for antibody responses and antifertility effects of MBP-mPH20. This method was chosen as it has previously been shown to induce high antibody titres to self-antigens in mice (Hardy & Mobbs 1999, Hardy *et al.* 2002*a*,*b*).

BALB/c mice (6–8 weeks old) were injected i.p. with 20 μg MBP-mPH20 (*n* = 10 female, 5 male) or MBP controls (*n* = 10 female, 5 male) in 100 μl 50% Freund's complete adjuvant (FCA) (Sigma). The animals were then boosted i.p. three times with 20 μg MBP-mPH20 or MBP in 100 μl 50% Freund's incomplete adjuvant (FIA) at 2-week intervals. Mice were bled from the suborbital sinus immediately prior to the first injection and again 2 weeks after the final boost. Serum was stored at − 20 °C. Pairs of treated female mice were coupled 2 weeks after the final immunization, when antibody titres were expected to be highest (Hardy & Mobbs 1999, Hardy *et al.* 2002*a*,*b*), with single males of proven fertility. Pairs of untreated females were added to each of the treated males 2 weeks after the final immunization. All males were removed after 2 weeks, and the females were

autopsied after a further 2 days to determine the number of live and dead embryos.

Immunization and mating protocols with rMCMV

The Animal Experimentation and Ethics Committee of the University of Western Australia approved animal experimental procedures with rMCMV, in compliance with the NHMRC guidelines.

BALB/c mice (6–8 weeks old) were inoculated i.p. with 2×10^4 plaque-forming units (pfu) of tissue culture-passaged rMCMV-mPH20 ($n = 6$ male, 6 female) or MOBS diluent containing 0.5% FCS ($n = 6$ female). Mice were paired immediately with uninfected animals of the opposite sex, and the total number of pups produced by each pair over the following 150 days (from up to seven litters) was counted in order to establish when infertility, if any, was present. All pups were killed immediately after birth by cervical dislocation. All parental mice were bled by cardiac puncture after the completion of the experiment and the serum was stored at $-20\,^{\circ}$C.

Assay of antibodies

Serum IgG antibody levels against recombinant antigens were determined by ELISA using 0.5 µg purified recombinant GST-mPH20, MBP-mPH20 or MBP protein per well as antigen, as previously described (Hardy *et al.* 2003). ELISA results for recombinant proteins are given as absorbance readings at 405 nm. Results for each serum dilution were accepted as positive where the absorbance reading after background subtraction was greater than 0.2, which was double the maximum absorbance observed for individual pre-immune sera in any of the assays. Serum IgG antibody responses against MCMV were determined as serum end-point titres by ELISA using 7.5 µg MCMV protein extract per well as antigen, as previously described (Lloyd *et al.* 2003). ELISA results for MCMV are given as serum end-point titres at 405 nm. Results for each serum dilution were accepted as positive where the absorbance reading was greater than the mean of normal mouse serum plus three times the standard deviation.

Protein extracts and Western blot analysis

SDS protein extracts of mouse caudal epididymal sperm were produced as previously described (Hardy & Mobbs 1999). Polyacrylamide gel electrophoresis and immunoblot analyses were conducted as previously described (Laemmli 1970), and proteins were transferred to Polyscreen PVDF transfer membranes (NEN DuPont, Boston, MA, USA) and probed with mouse sera diluted 1:100, followed by peroxidase-conjugated goat anti-mouse IgG secondary antibody diluted according to the manufacturer's recommendations (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Results are presented as means±S.E.M. Data were analysed by Student's *t*-test using SYSTAT (Evanston, IL, USA). A value of $P < 0.05$ was accepted as significant.

Results

Characterization of mPH20

The mPH20 (SPAM1) was cloned using reverse transcriptase PCR from the testis of BALB/c mice (submitted to GenBank, accession number AY228460). Several independent PCR products were sequenced and shown to have the identical sequence. The PCR product contained the entire coding region for mPH20 and included 24 nucleotides of $3'$ non-coding sequence. The sequence was identical to that present in three exons of a genomic mouse clone from chromosome 6 of strain C57BL/6J (GenBank accession number AC127559), but differed slightly from the cDNA sequences of a C57BL/6J and an unidentified mouse strain. The mPH20 coding region of BALB/c mice has a single nucleotide difference leading to one amino acid change relative to the sequence for SPAM1 cDNA isolated from a C57BL/6J mouse (GenBank accession number AK005638) and four nucleotide differences, leading to two amino acid changes, relative to the sequence from the undescribed strain of mouse (GenBank accession number U33958). Differences in the AY228460 mPH20 cDNA sequence relative to U33958 were nt 603 = T (vs C), nt 856–857 = GA (vs AG, expressed as glutamate vs arginine respectively), and nt 883 = T (vs C, expressed as phenylalanine vs leucine respectively). The difference in AY228460 relative to AK005638 was nt 1799 = A (vs G, expressed as histidine vs arginine respectively).

Properties of MBP-mPH20

Mouse PH20 was expressed with the N-terminus (from amino acid 30) fused to the C-terminus of maltose-binding protein (MBP-mPH20) in *E. coli* strains XL1-Blue and BL21-CodonPlus-RIL. The predicted size of the MBP-mPH20 fusion protein was 864 aa with an estimated molecular mass of 97 kDa. Initial studies of MBP-mPH20 protein expression were conducted in XL1-Blue cells, but the size of the recombinant protein was less than predicted, and it also appeared degraded. In contrast, the apparent molecular mass of the MBP-mPH20 protein on SDS–PAGE gels (97 kDa) was similar to the predicted size when expressed in BL21-CodonPlus-RIL cells (Fig. 1). Recombinant MBP-mPH20 protein prepared from BL21-CodonPlus-RIL cells was used in immunization and fertility trials.

Properties of recombinant GST-mPH20

Mouse PH20 was expressed with the N-terminus (from amino acid 30) fused to the carboxyl terminus of
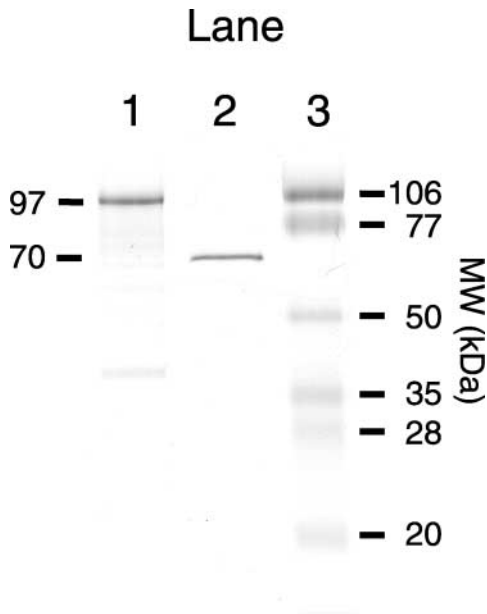
Lane

Figure 1 SDS–PAGE of affinity purified recombinant mPH20 proteins. Proteins were reduced and separated on a 12% SDS–PAGE gel and stained with Coomassie brilliant blue. Lane 1, MBP-mPH20; lane 2, GST-mPH20; lane 3, molecular mass markers. MBP-mPH20: mouse PH20 fused to maltose-binding protein; GST-mPH20: mouse PH20 fused to glutathione-*S*-transferase.

glutathione-*S*-transferase (GST-mPH20) in *E. coli* strain BL21-CodonPlus-RIL. The predicted size of the GST-mPH20 fusion protein was 724 aa with an estimated molecular mass of 83 kDa. The majority of the
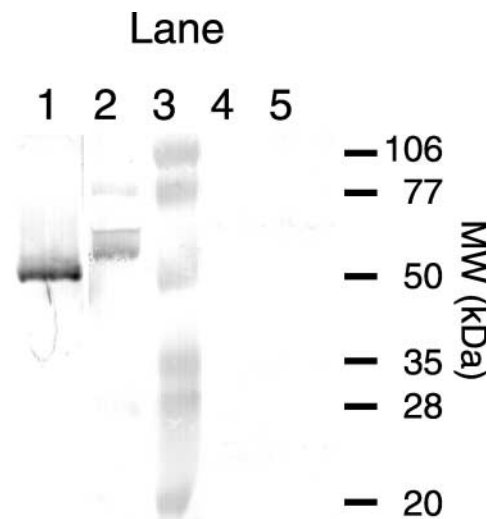
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Figure 2 Antibodies to mPH20 detected by Western blot. Protein extracts were reduced and separated on a 12% SDS–PAGE gel and transferred to PVDF membranes. Lanes 1, 4: SDS extracts of mouse sperm; lanes 2, 5: purified GST-mPH20. Membranes were probed with sera (diluted 1:100) raised in mice and visualized using horseradish peroxidase-conjugated goat anti-mouse IgG antibodies. Lanes 1, 2: MBP-mPH20 immune sera; lanes 4, 5 pre-immune sera; lane 3: molecular mass markers. MBP-mPH20: mouse PH20 fused to maltose-binding protein; GST-mPH20: mouse PH20 fused to glutathione-*S*-transferase.

recombinant GST-mPH20 protein appeared as a 70 kDa band on SDS–PAGE gels, 13 kDa less than predicted from the primary amino acid sequence (Fig. 1). However, some proteins were also detected at 60 and 80 kDa on Western blots (Fig. 2). Recombinant GST-mPH20 protein prepared from BL21-CodonPlus-RIL cells was used for ELISA and in Western blot analyses.

Restriction fragment length polymorphism of rMCMV expressing mPH20

Restriction fragment length polymorphism and Southern blot were used to verify the structure of the rMCMV-mPH20 virus (Fig. 3). Restriction digests were conducted using either HindIII or EcoRV, and probed using a 2672 bp NotI DNA fragment of pCMH158 containing the mPH20 cDNA. Insertion of the mPH20 gene expression cassette in the correct orientation into the *ie2* region of the HindIII L fragment of the MCMV strain K181 genome (expression in the same direction as the disrupted *ie2* gene) was
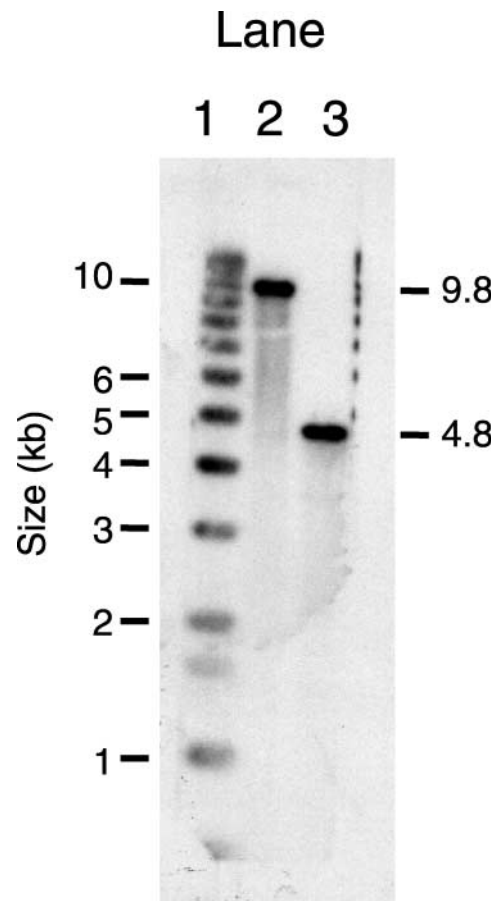
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Figure 3 Restriction fragment length polymorphism analysis of rMCMV-mPH20 viral DNA. Restriction enzyme digests of purified rMCMV-mPH20 DNA were separated on a 0.9% agarose gel, transferred to nylon membranes and probed with ^{32}P-labelled mPH20 DNA. Lane 1, 1 kb DNA marker; lane 2, HindIII digest; lane 3, EcoRV digest. rMCMV-mPH20: recombinant murine cytomegalovirus expressing mPH20.

confirmed by detection of DNA fragments with the anticipated sizes for both the HindIII (9762 bp) and EcoRV (4835 bp) digests of the viral DNA (Fig. 3).

Serum IgG responses of mice immunized with MBP-mPH20

Sera from mice inoculated with MBP-mPH20 were tested for the presence of cross-reactive IgG antibodies to recombinant GST-mPH20 by ELISA (Fig. 4) and to recombinant GST-mPH20 and sperm on Western blots (Table 1). No cross-reacting antibodies were detected by either ELISA (absorbance <0.1) or Western blot (no visible bands) against MBP, GST or GST-mPH20 in pre-immune serum samples diluted 1:100. In addition, no cross-reacting IgG antibodies against GST were detected by either ELISA or Western blot from any of the sera of treated mice at 2 weeks after the final immunization. There was considerable variation in the mPH20-specific serum antibody responses of individual BALB/c mice immunized with MBP-mPH20 using both detection methods.

The sera of all male and female mice immunized with MBP-mPH20 contained cross-reacting IgG antibodies to GST-mPH20 (absorbance >0.2) at 2 weeks after the final inoculation as determined by ELISA (Fig. 4). Absorbances at serum dilutions of 1:100 (the minimum
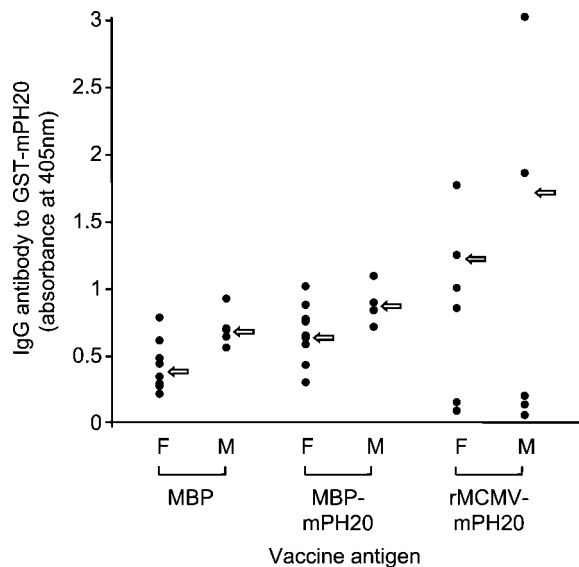

Figure 4 Antibodies to mPH20 detected by ELISA. Sera (diluted 1:100) from male and female mice were tested for the presence of mPH20-specific IgG antibodies by ELISA either 2 weeks after the final immunization (MBP and MBP-mPH20 treated mice), or 150 days after inoculation (rMCMV-mPH20 treated mice) using purified recombinant GST-mPH20 as detection antigen. GST-mPH20: mouse PH20 fused to glutathione-*S*-transferase; MBP: maltose-binding protein; MBP-mPH20: mouse PH20 fused to MBP; rMCMV-mPH20: recombinant murine cytomegalovirus expressing mouse PH20; F, females; M, males. Arrows indicate the mean of absorbance values at 405 nm from responder (absorbance >0.2) mice for each of the six groups of sera.

Table 1 Prevalence of seropositive sera by Western blot in BALB/c mice treated with recombinant mPH20 vaccines.

Treatment	Sex	Total number of mice	Number of seropositive mice[a]		
			Sperm	GST-mPH20	Sperm + GSTmPH20
MBP	Female	10	10	0	0
MBP	Male	5	2	0	0
MBP-mPH20	Female	10	10	6	6
MBP-mPH20	Male	5	4	4	4
rMCMV-mPH20	Female	6	1	4	1
rMCMV-mPH20	Male	6	1	3	0

[a] Sera were collected 2 weeks after the final boost (MBP, MBP-mPH20) or 150 days after infection (rMCMV-mPH20). Mice were scored as seropositive if bands were visible on Western blots probed with 1:100 diluted sera.

dilution tested) against GST-mPH20 ranged from 0.28 to 0.99 for females and 0.72 to 1.05 for males, with mean ODs of 0.61 ± 0.06 S.E.M. for females (MBP control 0.37 ± 0.06) and 0.85 ± 0.07 S.E.M. for males (MBP control 0.66 ± 0.06). All mice responded strongly to the MBP carrier protein, and antibodies to MBP (absorbance >0.2) were present in the sera of all MBP-mPH20 and MBP immunized mice at serum dilutions of up to 1:100 000. Cross-reacting IgG antibodies to both GST-mPH20 and sperm protein extracts were detected on Western blots in the sera (diluted 1:100) of 6/10 female and 4/5 male mice immunized with MBP-mPH20 (Table 1). All positive sera from the mice cross-reacted strongly to a 55 kDa protein in sperm extracts and up to three bands with apparent molecular masses of 60, 70 and approximately 80 kDa in purified GST-mPH20 (Fig. 2). None of the sera from control mice immunized with MBP alone cross-reacted to GST-mPH20 on Western blots. However, 12/15 of the immune serum samples from the MBP immunized mice cross-reacted with an ~ 55 kDa band in Western blots of sperm extract, although the band was faint (result not shown). Pre-immune sera from the MBP-mPH20 and MBP control mice did not react to either antigen by Western blot.

Serum IgG responses of mice inoculated with rMCMV-mPH20

Sera from mice inoculated with rMCMV-mPH20 were tested for the presence of cross-reactive IgG antibodies to recombinant GST-mPH20 by ELISA (Fig. 4) and to recombinant GST-mPH20 and sperm proteins on Western blots (Table 1). Sera from several mice (2/6 males and 2/6 females) did not contain IgG antibodies to mPH20 using any of the detection systems at 150 days post-inoculation, and serum from one male contained antibodies that cross-reacted only to sperm. However, cross-reacting antibodies were detected to GST-mPH20 by ELISA (absorbance >0.2) in the sera of the remaining male (3/6) and female (4/6) mice (Fig. 4) at 150 days post-inoculation. The mean

absorbances at serum dilutions of 1:100 against GST-mPH20 for these seropositive mice were 1.20 ± 0.20s.e.m. for females and 1.68 ± 0.22s.e.m. for males. The sera from the seven mice that reacted with GST-mPH20 by ELISA also reacted with GST-mPH20 by Western blot (Table 1). However, IgG antibodies that cross-reacted with a sperm protein band of 55 kDa on Western blots were detected only in the sera of two mice (one female and one male). Most mice (6/6 male and 5/6 female) inoculated with rMCMV-mPH20 produced detectable IgG antibody responses against MCMV by ELISA at 150 days after inoculation. The males produced generally higher levels of IgG antibodies (mean titre of $1:670 \pm 218$s.e.m. with a range of 1:20–1:1280) than the five positive females (mean titre of $1:22 \pm 7$s.e.m. with a range of 1:10–1:40).

Fertility of mice immunized with MBP-mPH20

There was no significant reduction in the mean litter sizes of female mice immunized with MBP-mPH20 and mated to untreated males, or of untreated females mated to male mice immunized with MBP-mPH20, compared with the control mice immunized with MBP alone (Table 2). The fertility data for the control MBP-treated group of female mice have been reported previously (Hardy *et al.* 2002*a*).

There was no correlation between fertility and the level of antibody response, either against sperm protein extracts by Western blot or against recombinant GST-mPH20 by ELISA of mice immunized with MBP-mPH20 (result not shown). The only significant difference ($P = 0.02$) was that MBP-treated males sired significantly fewer pups ($n = 26$) than the MBP-mPH20-treated males ($n = 61$) (Table 2).

Fertility of mice immunized with rMCMV-mPH20

There was no significant reduction in the fertility, mean litter sizes or total number of pups produced over 150 days for female BALB/c mice inoculated with rMCMV-mPH20 compared with females inoculated with diluent alone (Table 3). Likewise, there was no reduction in the fertility, mean litter sizes or total number of pups in untreated females mated with rMCMV-mPH20-inoculated males compared with diluent controls. All six male and six female BALB/c mice that were inoculated with rMCMV-mPH20 and five of six female BALB/c mice treated with MOBS diluent produced litters during the 150 days after inoculation (Table 3).

There was no correlation between the number of pups born to individual parents and the presence or absence of IgG antibody responses against mPH20 either by ELISA or by Western blot in mice inoculated with rMCMV-mPH20 (result not shown).

Discussion

The development of PH20 as the basis for contraceptive wildlife vaccines relies on the generation of appropriate sources of antigen and delivery mechanisms (Holland *et al.* 1997 , Pomering *et al.* 2002). Two different antigen and delivery formulations of recombinant mouse PH20 (mPH20, SPAM-1) were therefore produced as part of an effort to develop immunocontraceptive vaccines for use as biological control agents for mice. The first vaccine was in the form of repeated direct injections of mice with mPH20 produced in a bacterial expression system (MBP-mPH20), and the second was a single inoculation of mice

Table 2 Fertility of BALB/c mice immunized with recombinant MBP-mPH20 protein.

Antigen	Sex	Number of mice	Number of fertile mice	Number of implantations	Number of viable embryos	Litter size (mean± s.e.m.)
MBP-mPH20	Female	10	8	45	45	4.5 ± 1.3
MBP[a]	Female	10	7	55	52	5.2 ± 1.3
MBP-mPH20	Male	5	5	67	61[b]	6.1 ± 1.2[c]
MBP	Male	5	2	27	26[b]	2.6 ± 1.2[c]

[a] Fertility data for the group of MBP–treated females have been published (Hardy *et al.* 2002*a*).
[b] Embryos produced from ten untreated females mated to five treated males.
[c] There was significant difference between the mean number of pups sired by male mice treated with MBP and MBP-mPH20 ($P = 0.02$). There was no significant difference between the mean litter sizes of the treated and control groups of female mice.

Table 3 Fertility of BALB/c mice inoculated with rMCMV-mPH20.

Inoculum	Sex	Number of mice	Number of fertile mice	Total litters	Live pups	Total pups[a]	Litter size (mean± s.e.m.)[a]
rMCMV-mPH20	Female	6	6	32	201	207	6.5 ± 0.5
rMCMV-mPH20	Male	6	6	30	203	207	6.9 ± 0.4
MOBS	Female	6	5	26	156	158	6.1 ± 0.5

[a] Total number of pups produced over 150 days after inoculation, including dead pups. No significant difference in production of pups was present between the treated and control groups of mice.

with a recombinant MCMV expressing mPH20 (rMCMV-mPH20). Neither recombinant mPH20 vaccine formulation significantly reduced the fertility of either female or male mice.

Several factors may have contributed to the inability of the rMCMV-mPH20 or MBP-mPH20 to cause infertility in mice. In particular, native PH20 proteins may have antigenic epitopes with immunocontraceptive potential not present in either of the recombinant forms used in this study. For example, native PH20 is proteolytically cleaved late in sperm maturation during passage in the epididymis in rodents such as guinea pigs (Phelps *et al.* 1990), rats (Seaton *et al.* 2000) and mice (Deng *et al.* 1999). In addition, modifications to N-linked oligosaccharides on native mPH20 have also been reported during the passage of mouse sperm through the epididymis (Deng *et al.* 1999). Neither of these modifications is likely to have occurred to the recombinant proteins. It is therefore possible that such epididymis-specific modifications to PH20 are required to provide B-cell epitopes associated with the immunocontraceptive effects seen in guinea pigs with native gpPH20 isolated from sperm (Primakoff *et al.* 1988, 1997, Tung *et al.* 1997). In this respect, there is evidence that antisera produced by BALB/c mice to MBP-mPH20 and rMCMV-mPH20 cross-reacted to only one, partially glycosylated form of mPH20. The mice treated with the MBP-mPH20 and rMCMV-mPH20 vaccine formulations that were seropositive on Western blots of BALB/c caudal sperm all recognized a band with apparent molecular mass of ~55 kDa. This is smaller than the principal band of ~67 kDa previously reported by others for mPH20 (SPAM-1), but similar to a minor band present in extracts of acrosome intact caudal sperm (Thaler & Cardullo 1995, Deng *et al.* 1999, 2000, Zhang & Martin-DeLeon 2001, Zhang & Martin-DeLeon 2003*a*). The discrepancy in the estimated sizes of the mPH20 sperm protein may be explained by differences in the SDS–PAGE protocols and molecular mass markers used, although the major band detected in extracts of acrosome-reacted sperm and partially de-glycosylated sperm is reported to be 56 kDa (Deng *et al.* 1999).

Interestingly, an ~55 kDa band in sperm was also recognized by sera in some mice immunized with MBP alone. Although we cannot discount the possibility that the cross-reaction of antibodies to sperm was due to MBP-specific antibodies in the mice, the intensity of the reaction on Western blots was considerably greater for mice immunized with MBP-mPH20 than for those immunized with MBP alone (result not shown). This suggests that mPH20-specific antibodies were indeed produced by the MBP-mPH20-immunized mice and that the antibodies in MBP-immunized mice that reacted with the sperm extract were to another protein of similar molecular mass. The cross-reactivity of rabbit serum containing MBP-specific antibodies to sperm has also been noted in rabbits (Hardy *et al.* 1997).

The MBP-mPH20 and rMCMV-mPH20 vaccines induced qualitatively different mPH20-specific antibodies in the mice when tested against non-denatured (ELISA) or denatured (Western blot) mPH20 proteins. For example, individual mice inoculated with rMCMV-mPH20, but not MBP-mPH20, appeared to be either responders ($n = 7$) or non-responders to GST-mPH20 ($n = 5$). Only 3/6 males and 4/6 females inoculated with rMCMV-mPH20 exhibited GST-mPH20-specific antibodies at serum dilutions of 1:100. In addition, antibodies that cross-reacted to both GST-mPH20 and sperm on Western blots were present in the sera of most mice (10/15) immunized with MBP-mPH20, compared with only a single mouse inoculated with rMCMV-mPH20 (Table 1). The different immune responses can be attributed to differences in the structural features of the two recombinant mPH20 antigens. In particular, post-translational modifications, such as glycosylation of mPH20, may have taken place in the virally expressed protein, but not in the bacterially produced fusion protein (Benz & Schmidt 2002). However, regardless of the theoretical differences in the glycosylation of the PH20 protein in the two vaccine formulations, an immunocontraceptive effect was not apparent for either. This again may indicate that the native mPH20 protein could contain critical immunocontraceptive B-cell epitopes that were not present in the recombinant forms.

Lack of immunocontraceptive effect for PH20 has also been reported for bacterially produced rabbit PH20 in rabbits (Holland *et al.* 1997), and in this animal, the inability to cause sterility was directly attributed to insufficient PH20 antibodies in the male and female reproductive tracts (Pomering *et al.* 2002) rather than to absence of critical epitopes in the protein. Therefore, there may have been insufficient mPH20 antibodies generated in the reproductive tracts to cause infertility in mice using the immunization regimes of the present study. In this respect, effects on fertility of female BALB/c mice immunized with another recombinant sperm antigen (sp56) were seen only after the mice had received five, and not three, boosts (the regime used for MBP-mPH20) with the protein in Freund's incomplete adjuvant (Hardy & Mobbs 1999).

In guinea pigs, infertility following immunization with a single injection of native gpPH20 was reversible (Primakoff *et al.* 1988, 1997, Tung *et al.* 1997). These studies were conducted in outbred guinea pigs, and although all the animals developed high titres of PH20-specific antibodies, recovery of fertility could not be correlated with any reduction in antibody titres. Therefore, it is possible that appropriate PH20-specific cellular immune responses are required for development of contraceptive responses and that the inbred BALB/c mice used in the present study did not produce these responses. In this respect, antisera raised in BALB/c mice against BALB/c sperm have previously been shown to contain far fewer sperm-reactive antibodies than antisera raised in BALB/c mice against sperm from other strains of mice (Xu & Anderson 1987). Conversely, significant immune reactions and effects on

fertility in mice have been shown to occur after passive transfer of leucocytes from mice immunized with whole mouse sperm, but only when the leucocytes were produced in a non-identical strain (Tinneberg *et al.* 1980). Examination of the GenBank database revealed that amino acid polymorphisms are present between mPH20 sequences from different mouse strains, and it is possible that immunization of BALB/c mice with recombinant mPH20 proteins derived from another strain of mice could improve cellular immunogenicity and produce a contraceptive effect.

The strong immunocontraceptive effect reported for gpPH20 in guinea pigs (Primakoff *et al.* 1988, 1997, Tung *et al.* 1997), but not seen for mice in this study, could reflect more fundamental differences in the biological role of PH20 between the two species. It has been shown recently that mPH20 gene knockout mice remain fully fertile and that mPH20 is dispensable for fertilization and sperm penetration of cumulus in mice (Baba *et al.* 2002). Although this does not preclude the ability of an anti-PH20 immune response to disrupt fertilization, it suggests that PH20 may not play as essential a role in fertilization in mice as it does in guinea pigs. PH20 appears to be expressed in the male reproductive tract only of several species, including guinea pig (Primakoff *et al.* 1988), man (Lin *et al.* 1993) and rabbit (Holland *et al.* 1997), whereas mPH20 (SPAM-1) expression is more widespread. PH20 protein and transcripts have been identified in testis, epididymis and kidney cells (Deng *et al.* 1999, 2000, Zhang & Martin-DeLeon 2001, Zhang & Martin-DeLeon 2003*a*), as well as in the female reproductive tract (Zhang & Martin-DeLeon 2003*b*). The more widespread expression of mPH20 in mice could also affect the ability to break tolerance to PH20 by active immunization in this species.

Finally, the lack of contraceptive effect with mPH20 is unlikely to be due to limitations of the delivery systems. In contrast to the present data with mPH20, the female-specific antigen mZP3 induced infertility in female BALB/c mice when they were treated using the same vaccine approaches used for mPH20 (Hardy *et al.* 2003, Lloyd *et al.* 2003). In particular, rMCMV-mZP3 induced long-lasting sterility in almost 100% of female mice after a single inoculation (Lloyd *et al.* 2003).

In conclusion, fertility control using disseminating virally vectored contraceptive vaccines has the potential to provide an effective means for reducing pest animal populations, such as those of house mice, which cause significant economic damage in southern Australia. However, we have demonstrated that recombinant mPH20 appears to be unsuitable for use in contraceptive vaccines for mice when delivered either as a protein produced in bacteria or by rMCMV.

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