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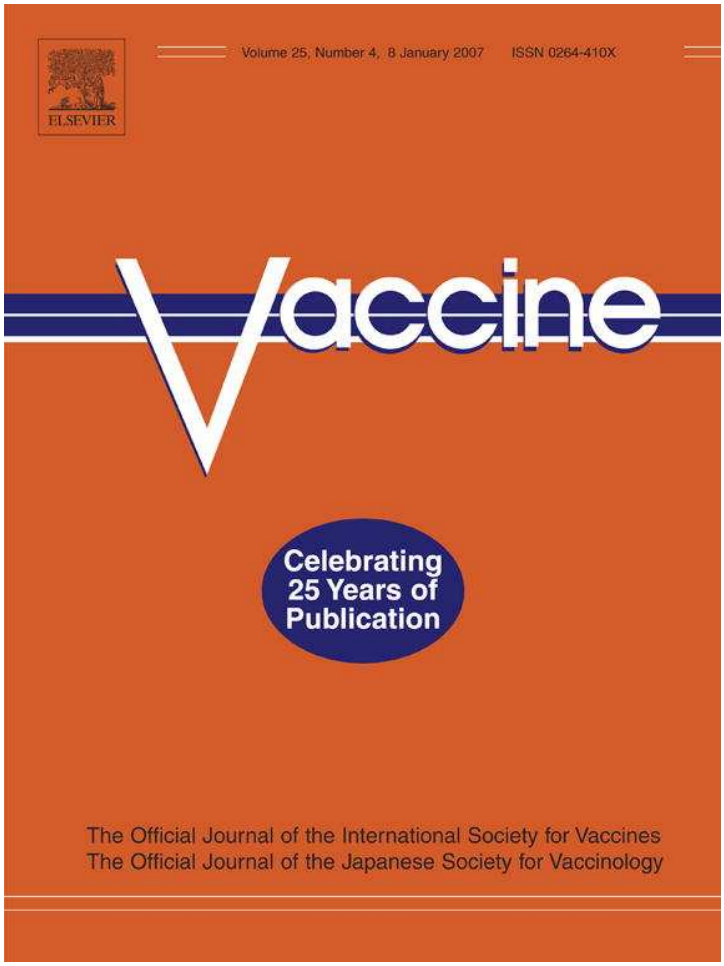

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Viral vectored immunocontraception: Screening of multiple fertility antigens using murine cytomegalovirus as a vaccine vector

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Abstract

Mouse cytomegalovirus (MCMV) has previously been used as a vaccine vector for viral vectored immunocontraception (VVIC). MCMV expressing murine zona pellucida 3 (mZP3) induces long term infertility in up to 100% of female BALB/c mice following a single inoculation. Whilst a large number of antigens have been investigated as potential immunocontraceptive vaccines, it has been difficult to compare these antigens as few studies have used identical approaches or even animal species. Here a range of protein and polyepitope antigens, all expressed by MCMV, were tested for the ability to sterilise female mice. The antigens tested were bone morphogenic protein 15 (BMP15), oviduct glycoprotein (OGP) and ubiquitin-tagged mZP3. In addition, four polyepitope constructs that contain rodent or mouse specific epitopes were tested. This study found that when expressed by an MCMV vector, only full-length mZP3 or ubiquitin-tagged mZP3 induced infertility in female mice. BMP15 and OGP had no effect. Of the four polyepitopes tested, one had a partial effect on fertility. These data indicate that while MCMV is an effective vector for VVIC, the antigen used needs to be tested empirically. The partial infertility seen in mice infected with one of the polyepitope vaccines is a promising finding suggesting that it may be possible to combine a species specific virus with a species specific antigen for use as a disseminating mouse control agent.
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Keywords: Cytomegalovirus; Zona pellucida; Polyepitope; Bone morphogenic protein 15; Oviduct glycoprotein

1. Introduction

A wide range of viruses have been identified as potential vaccine vectors, including poliovirus [1,2], vaccinia virus [3], canarypox [4,5], rabies virus [6], adenovirus [7] and herpesviruses [8,9]. Viral vectors have been used to vaccinate against infectious agents [6,7], malignancies [2,5] and induce immunocontraception [10–13]. Viral vectors deliver the antigen directly into host cells, allowing high-level intracellular expression. In addition, because the immune system has evolved a sophisticated array of mechanisms to detect and respond to the antigens of invading viruses they may serve as an adjuvant for the expressed antigen.

We have previously used the herpesvirus, murine cytomegalovirus (MCMV) as a vaccine vector and have reported its use as a viral vectored immunocontraceptive (VVIC) [11,12]. CMVs are members of the β-herpesvirinae of the Herpesviridae and contain a large double stranded DNA genome of approximately 230 Kb [14]. CMVs are found in a broad range of mammalian species but exhibit strict species specificity [15]. They cause asymptomatic infections in immunocompetent animals and persist in a latent state for the lifetime of the host. Their properties of latency, large DNA size and strict species specificity make CMVs useful vac-

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cine vectors. CMVs induce strong and long-lived cytotoxic T lymphocyte (CTL) responses [16,17], as well as long-lived antibody responses [18]. It is likely that the persistence and reactivation of MCMV contributes to the long-lived immune responses to MCMV. The ability to clone herpesviruses [19,20], including cytomegaloviruses, as bacterial artificial chromosomes has added to their vaccine potential [12,21].

The success of any vaccination process is dependent not only on the delivery system, in this case the viral vector, but also on the antigen that is delivered. For immunocontraception, the choice of antigen is of particular relevance as the aim of this immunization protocol is to break self-tolerance. This may be easier with some self-antigens than others, as autoantigens are generally restricted to a limited range of proteins [22]. However, breaking self-tolerance may not be sufficient for immunocontraception, as autoimmune responses are not necessarily linked to altered physiology [22]. Consequently, for immunocontraception it is necessary to break tolerance, and for the response to be sufficient in quantity or quality to result in infertility. This could be in the production of an inflammatory response that inhibits normal function in an important organ, such as the ovary or could be due to complete or partial loss of an antigen that is essential for reproduction [23,24], such as zona pellucida 3 (ZP3). Given these complexities it is not possible to determine, except empirically, which antigens will make a suitable target for immunocontraception. Consequently, we chose to test a range of antigens associated with female reproductive processes to determine if immune responses to one or more were capable of sterilising female mice.

Full-length protein antigens were tested as alternatives or improvements on the previously successful mZP3 antigen [11,12]. Possible alternative proteins tested were BMP15 and OGP, both of which are expressed within the reproductive system of female mice [25–27]. The mZP3 antigen was N-terminal ubiquitinated and tested as an improvement on mZ3 alone. Ubiquitination has been shown to improve $CD8^+$ T cell responses and MHC class I antigen processing by targeting proteins to the proteosome [28,29]. Finally, polyepitope antigens were tested in the MCMV vector for the capacity to sterilise female mice. These polyepitope antigens have been previously assessed as immunocontraceptive vaccines when co-administered with adjuvant [30,31] and were included in this study as they contain mouse or rodent specific epitopes. The expression of these polyepitopes by MCMV may enhance the safety of VVIC for widespread use [32] by combining a species specific vector, MCMV, with a species specific antigen.

To allow comparison between the antigens tested, the same MCMV vector, K181, was used for each antigen with the same insertion site, the non-essential *ie2* gene [33]. The expression of each antigen was driven by the HCMV *ie1* (UL123) gene promoter. All infection conditions were identical. We find that the capacity to induce infertility is dependent on the antigen chosen, and that of those tested, only those expressing mZP3, a ubiquitin-tagged mZP3 or the polyepitope B affected the fertility of female BALB/c mice. No demonstrable effect on the fertility of female mice was seen with the viruses expressing mRNA for BMP15, OGP or the polyepitopes A, C or Z.

2. Materials and methods

2.1. Virus and cells

The origins of the K181 strain of MCMV have been described previously [34]. The virus $RM427^+$ was kindly provided by Professor E. Mocarski (Stanford University, Stanford, USA). Virus stocks were propagated in mouse embryonic fibroblasts (MEF) as previously described [35]. Viral titres were determined in duplicate by plaque assay in MEF [11].

2.2. Animals

Specific pathogen-free BALB/c mice were obtained from the Animal Resource Centre (Murdoch, Western Australia) and housed under minimal disease conditions. Mouse care was based on the Australian Code of Practice and was approved by the University of Western Australia Animal Experimentation and Ethics Committee. Sentinel animals were found to be free of a suite of murine pathogens including MCMV following routine testing.

2.3. RNA purification and PCR conditions

Total RNA was prepared as previously described [36] from mouse testes, ovaries or oviducts and polyadenylated RNA was isolated using a PolyAttract System III kit (Promega, Madison, Wisconsin, USA) and reverse transcribed into cDNA using a TimeSaver cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden) with either the pd(T12–18) and pd(N6) primers provided in the kit or gene specific primers. PCR was conducted in 50–100 μl total volumes containing 1–5 ng of DNA, 1–2 μM of each primer, 200 μM of dNTPs, and either 1 unit of *Pfu* (Stratagene, La Jolla, CA) or 2.5 units of *Taq* (Promega) DNA polymerase added "hot start" in the reaction buffers provided by the manufacturers. Nested PCRs were conducted using 1 μl of the first PCR reaction as template.

2.4. Cloning of mouse BMP15 and OGP

Mouse BMP15 (GenBank accession number AF082348) was obtained by nested PCR on BALB/c ovary cDNA reverse transcribed with pd(T12–18) primers. Primers B15F1, B15R1 and B15F2, B15R2 (Table 1) were used for the first and second nested PCRs, respectively. The resulting 1220 bp PCR product was cloned into pGEM-T-easy (Promega, Madison, Wisconsin, USA) to produce plasmid pCMH264. Mouse OGP (GenBank accession number AY521455) was obtained

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Table 1
Oligonucleotides used for PCR and direct annealing

Oligonucleotide	DNA sequence (5′–3′)[a]	DNA sequence location[b]
B15F1	GACTTGGTTAGGAATCTGCTG	316–336 (NM_009757)
B15F2	gccaccATGGCCCTTCTCACAATTC	358–376 (NM_009757)
B15R1	TCACTGCCAGCTTTAACACAG	1613–1593 (NM_009757)
B15R2	TATAGCTTCTTGGGAAACCTG	1577–1557 (NM_009757)
hTFRF3	ttgagctcCATCGGGTGGCGGCTCG	38–53 (M11507)
hTFRR4	tcggatccGAAGTCCTCTCCTGGCTC	443–426 (M11507)
RLF3	tgtctagaGACGTGGACCCAGCGCTA	234–251 (S82815)
RLF4	tgactagtACTGCGGCGCTGGCGCT	296–280 (S82815)
mTFR1	tggtcgacATGATGGATCAAGCCAG	50–66 (NM_011638)
mTFR2	tgggatccCTCTGTTTCCATGGTTTC	397–380 (NM_011638)
MUBF	gccaccATGCAGATCTTCGTGAAG	105–122 (X51703)
MUBR	ggatccggcACCTCTCAGGCGAAG	329–315 (X51703)
Ovi1	ccgtcgacAATAGAGCTGGAAGACC	2201–2185 (D32137)
Ovi2	cactcGAGATGGGGAGGCTGCT	12–28 (D32137)
Ovi3	tgactagtCATTGTGGCTGTGGTC	1961–1945 (D32137)
PLGA	ctagaAGGAGTAGAAACTCACAGACTGAGGAACCCCACCCGtaag	756–791 (X96793)
PLGB	tcgacttaCGGGTGGGGTTCCTCAGTCTGTGAGTTTCTACTCCTt	791–756 (X96793)
Ovi4	tgctcgagATGAGCACACTGGACTT	1449–1465 (D32137)
LK1	P-GCGGCCGCTCTGGCGGT	
LK2	P-CCGCCAGAGCGGCCGC	
OGP F	TCCTTCCTGAGAATACATGG	390–409 (D32137)
OGP R	GCGCATCATAAGACGTATGG	609–590 (D32137)
bmp15 F	CCTGGACTTTTCCTCTAGCAT	699–718 (AF82348)
BMP15 R	GAAGAACACTCCGTCCCTTG	961–942 (AF82348)
Univ TRF F	CATATACCCGGTTCAGCCTG	151–170 (M11507)
Uni AgA-C R	GCCCAAGTAGCCAATCATAA	356–337 (M11507)
TFR anchor F	GATGAAACTGGCTGCAGATG	160–179 (NM_011638)
AgZ R	ACATCTAGACCTGCGGTTTC	Plasmid pCMH450
ZP3-380F	ACCCTCGCCCTGTGAGTGGCC	382–402 (M20026)
ZP3-1021R	AATTACTACAGTTGCCATGGC	1021–1000 (M20026)
Im2AF	CATTAAAAACTATTGGTTCTA	184196–184216 (NC_004065)
Im2AR	CCCATAGCCGAGCCCAATGCA	184375–184355 (NC_004065)

Primers and linkers used in the construction of plasmids and analysis of recombinant viruses.
[a] Lower case text indicates restriction enzyme sites or modifications included in oligonucleotides to facilitate sequential cloning or to introduce transcriptional stop signals.
[b] DNA sequence locations are given according to the nucleotide numbering for the designated GenBank accession numbers.

by PCR using primers Ovi1, Ovi2 (Table 1) on BALB/c oviduct cDNA reverse transcribed with pd(N6) primers. The resulting 2161 bp PCR product was digested with *Xho*I and *Sal*I and cloned between the *Xho*I and *Sal*I sites of pBluescript KS− to produce pCMH113.

2.5. Cloning of mouse ZP3 fused to ubiquitin

The plasmid pCMH316 was produced by PCR amplification of mouse ubiquitin monomer (GenBank accession number X51703, ref. [37]) with primers MUBF and MUBR (Table 1) designed to include a KOZAC consensus sequence. A G26 to A26 amino acid substitution was included to produce a fusion protein according to Rodriguez and colleagues [29] with mZP3. The 240 bp ubiquitin PCR product was produced from BALB/c genomic DNA and cloned into pGEM-T-easy to produce the plasmid pCMH307. The plasmid pZP3 containing mZP3 cDNA has been described [10]. A 1308 bp *Bam*H1/*Xho*1 fragment containing the mZP3 cDNA insert in pZP3 was transferred into the *Bam*H1/*Xho*1 sites of the pFastbac HTb baculovirus expression vector (Gibco BRL) to produce pCMH119. The 1318 bp *Bam*HI/*Sph*I DNA fragment containing the mZP3 coding sequence from pCMH119 was inserted between *Bam*HI/*Sph*I of pCMH307 such that the mZP3 coding sequence was fused in frame with the ubiquitin monomer of pCMH307 to produce pCMH312.

2.6. Cloning of polyepitope antigen DNA sequences

The DNA sequence coding for the human transferrin receptor transmembrane signal and anchor domains (hTfR) was produced by PCR using the primer pair hTFRF3/hTFRR4 (Table 1) from plasmid pSVT7-Ova/TfR [38]. The DNA sequence coding for the mouse transmembrane signal and anchor domains [39] was obtained by PCR using the primer pair mTFR1/mTFR2 (Table 1) from Swiss mouse oviduct cDNA. The mouse specific DNA sequences for the relaxin like factor (RLF), also known as leydig insulin-like protein, were obtained by PCR using the primer pairs RLF3/RLF4 and Ovi3/Ovi4, respectively (Table 1) from BALB/c mouse genomic DNA. The mouse specific region

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Table 2
Mouse or rodent specific peptide component and arrangement in the polyepitopes in rK181-AgA, -AgB, -AgC and -AgZ

Polyepitope	Components	Accession #
Antigen A	hTfR, BRN-T, SP56, PLF, HA-T, ZP3-T, ZP3-B, PRL, GM-CSF	AY521451
Antigen B	hTfR, BRN-T, SP56, PLF, HA-T, ZP3-T, ZP3-B, ZP1	AY521452
Antigen C	hTfR, BRNT, OGP, HA-T, ZP3-T, ZP3-B, RLF, PLG	AY521453
Antigen Z	mTfR, furin, ZP3-T, ZP3-B (three repeats), ZP1	AY521454

Abbreviations: hTfR, human transferin receptor; mTfr, mouse transferin receptor; BRN-T, immunodominant T helper epitope from bovine ribonuclease; SP56, sperm protein 56; HA-T, immunodominant T helper epitope from influenza virus haemagglutinin; ZP3-T, mZP3 CD8$^+$ T cell epitope; ZP3-B, mZP3 B cell epitope; GM-CSF, granulocyte macrophage colony stimulating factor epitope; ZP1, mouse zona pellucida 1; OGP, oviduct glycoprotein; RLF, relaxin like factor; PLG, placental growth factor; PLF, proliferin; PRL, prolactin; furin, furin cleavage site.

of PLG was produced by annealing oligonucleotides PLGA and PLGB (Table 1).

The DNA constructs for polyepitope antigens AgA, AgB and AgZ have been described previously [30,31] and the peptide components are given in Table 2. AgC was assembled by sequential cloning of annealed oligomeric primers and PCR products into pGEM-T-easy and pBluescript SK− (Stratagene, La Jolla, CA, USA) cloning vectors. AgA (GenBank accession number AY521451), AgB (AY521452) and AgC (AY521453) were fused in frame after the hTfR sequence. AgZ was produced by fusing the polyepitope AgZ (GenBank accession number AY521454, ref. [30]) to the mTfR anchor sequence. Cloned inserts were confirmed by sequence analysis.

2.7. Construction of expression vectors

In all cases the antigen constructs described above were cloned into one of five expression vectors pMV11 ([11], pCMH148 (GenBank accession number AY122059), pCMH149 (AY122060), pCMH285 (GenBank accession number AF525778) or pCMH411 (GenBank accession number AY122058) (see Table 3). This cloning strategy resulted in expression cassettes that were flanked by *Not*I restriction enzyme sites for ease of cloning into the recombination plasmids.

2.8. Construction of recombination plasmids

The recombination plasmid pH3LN [11] contains the entire *Hin*dIII L fragment of MCMV, strain K181, inserted into plasmid pUC9 with the 79 bp *Hpa*I fragment replaced by a *Not*I linker (LK1/LK2 Table 1). All antigen expression cassettes were cloned into the recombination plasmid pH3LN using *Not*I restriction enzyme within the non-essential *ie2* gene such that transgene expression was in the same direction as the non-essential *ie2* gene (Table 3).

2.9. Generation of recombinant viruses in cell culture

Recombinant viruses were produced as previously described [11]. Briefly, 10–50 μg of RM427$^+$ DNA was co-transfected with 2–5 μg of the appropriate *Sac*I linearised plasmid (see Table 3) into 90% confluent MEF cultures by calcium phosphate precipitation (CellPhect) according to the manufactures instructions (Amersham Pharmacia Biotechnology UK Ltd., Buckinghamshire, UK). Cells were screened for the development of plaques and then stained for the expression of β-galactosidase by the addition of X-gal. White, non-staining, plaques were picked and purified by three sequential rounds of plaque purification. Recombinant viruses were screened by RFLP and the insertion site and antigen confirmed by sequencing across the insertion site using primers Im2AF and Im2AR (Table 1). Viral stocks were made for experimentation on MEF.

2.10. Viral transgene mRNA expression

All viruses were tested for transgene expression by reverse transcription (RT)-PCR. MEF were infected with virus at an MOI of 0.5 and allowed to replicate until 100% CPE was noted. Total RNA was extracted from virus infected cells

Table 3
Recombination plasmids used in the construction of recombinant viruses

Virus	Gene/polyepitope	Expression plasmid	Promoter	Terminator	Recombination plasmid
rK181-ZP3	mZP3	pMV11	HCMV ie1	HCMV ie1	pK181-H3L-MV11-ZP3
rK181-UbZP3[a]	mZP3	pCMH285	HCMV ie1	HCMV ie1	pCMH316
rK181-BMP15[a]	BMP15	pCMH148	HCMV ie1	SV40	pCMH275
rK181-OGP[a]	OGP	PCMH148	HCMV ie1	SV40	pCMH117
rK181-AgA[a]	AgA	PCMH148	HCMV ie1	SV40	PCMH245
rK181-AgB[a]	AgB	PCMH148	HCMV ie1	SV40	pCMH246
rK181-AgC[a]	AgC	PCMH149	HCMV ie1	SV40	pCMH262
rK181-AgZ[a]	AgZ	PCMH411	HCMV ie1	SV40	pCMH450

All plasmids were linearised with *Sca*I and co-transfected with RM427$^+$ DNA using a calcium phosphate precipitation method.
[a] Antigens cloned as *Not*I gene cassettes in the expression plasmids pMV11, pCMH148, pCMH149, pCMH285 and pCMH411. Antigen expression cassettes were subsequently transferred into the *Not*I site of the recombination plasmid pH3LN.

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using Trizol according to manufacture's instructions (Invitrogen, Carlsbad, CA). Viral mRNA was treated with RQ1 RNA free DNase (Promega) and reverse transcribed with AMV reverse transcriptase (Promega) with the addition of RNasin (Promega). Control samples were treated identically without the addition of AMV reverse transcriptase. Samples were subjected to PCR using the primers described in Table 1 for the viruses rK181-BMP15 (BMP15 F and BMP15 R), rK181-OGP (OGP F and OGP R), rK181-UbZP3 (mZP3/380F and mZP3/1021R), rK181-AgA, B, C (Uni AgA-C R and TFR R) and rK181-AgZ (TFR anchor F and AgZ R).

2.11. mZP3 ELISA

Sera from infected mice were tested by ELISA for the presence of antibody to mZP3. ELISA conditions were as previously detailed [11]. Briefly, seroconversion was assessed by adding serum from infected mice to ELISA plates coated with mZP3 antigen, with stepwise addition of biotinylated rat anti-mouse IgG1 or IgG2a (Southern Biotech Association, Inc., Birmingham, AL), streptavidin alkaline phosphatase (Amersham Biosciences, Piscataway, NJ) and p-nitrophenyl phosphate (Sigma, St. Louis, MO). Since MCMV induces a polyclonal B cell response [40,41]. The highest anti-mZP3 antibody titre found in the control K181 virus infected mice was chosen as the cut-off point for assessing the specific anti-mZP3 antibody responses in vaccine infected mice. Each plate contained a positive control of rK181-ZP3 hyperimmune serum and negative control, RM427$^+$, hyperimmune serum generated from mice inoculated three times with the appropriate virus.

2.12. Breeding trials

Female BALB/c mice were inoculated i.p. with 2×10^4 plaque forming units (PFU) of tissue culture derived rMCMV constructs. In each experiment a positive control, consisting of mice inoculated with rK181-ZP3, was included [11]. Negative control mice were inoculated with either parental K181 virus or diluent alone. The minimum number of females inoculated in any one treatment was five. Each female was paired with a single male immediately after inoculation. This methodology typically results in mice having a first litter and confirms the initial fertility of the female mouse. Males were rotated between females within a treatment group weekly to control for differences in male fertility. The breeding output (the number of pups born) over 100 days was recorded for each female mouse.

2.13. Statistics

In all cases only female mice that were proven fertile by having a first litter were included in further analyses. In addition, because the first litter was included as a control for fertility the breeding data in the text is expressed as the mean ± S.E.M. of the cumulative number of pups per female, excluding this first litter control. To account for differences in mouse numbers in each data set, the breeding success was expressed as cumulative mean number of pups per female. For ease of presentation the breeding data from the controls of all the individual experiments have been combined and is presented together in table or graph format. There were no significant differences between the replicates of any treatment, including controls, allowing compilation. One-way ANOVA, with Tukey's honestly significant post hoc test (SPSS), was used to compare experimental and control treatment results.

3. Results

3.1. Virus construction

A series of MCMV constructs expressing fertility antigens were produced and tested for their efficacy in sterilising female BALB/c mice. The protein antigens tested were; murine bone morphogenic protein 15 (BMP15), murine oviduct glycoprotein (OGP) and an N-terminal ubiquitin-tagged murine zona pellucida 3 (UbZP3). The polytopes that were tested contained epitopes of important immunological targets such as ZP1, ZP3, OGP and sperm protein SP56. Other peptide epitopes included; reproductive hormones such as relaxin like factor, placental growth factor (PLG), proliferin, prolactin and the immunostimulatory molecule granulocyte macrophage colony-stimulating factor (GM-CSF). These were included in various combinations into, AgA, AgB, AgC and AgZ (Table 2).

Fig. 1A demonstrates the cloning strategy adopted to produce the recombinant MCMV vectors. Shown is the parental K181 virus and the intermediate virus, RM427$^+$ (kindly provided by Prof. E. Mocarski), containing a LacZ cassette. For illustration purposes the fertility antigen shown in Fig. 1A is AgB, however all recombinant viruses were made in a similar manner. All recombinant viruses were screened by RFLP analysis (Fig. 1B and data not shown) and the correct insertion was confirmed by sequencing across the insertion site (data not shown). Fig. 1B demonstrates an RFLP of the recombinant MCMV (rMCMV) expressing AgB. In all cases recombinant viruses exhibited alterations to the size of the *Hin*dIII L fragment consistent with the insertion of the appropriate expression cassette.

Transgene expression from all virus constructs was verified by RT-PCR for the appropriate cDNA or polyepitope using insert specific primes (Table 1). All rMCMVs expressed mRNA for the appropriate cDNA (Fig. 1C and data not shown).

3.2. In vitro replication of recombinant viruses

All recombinant viruses displayed replication kinetics similar to both the parental strain of virus, K181 and to RM427$^+$ (Fig. 2). Given their normal in vitro growth charac-

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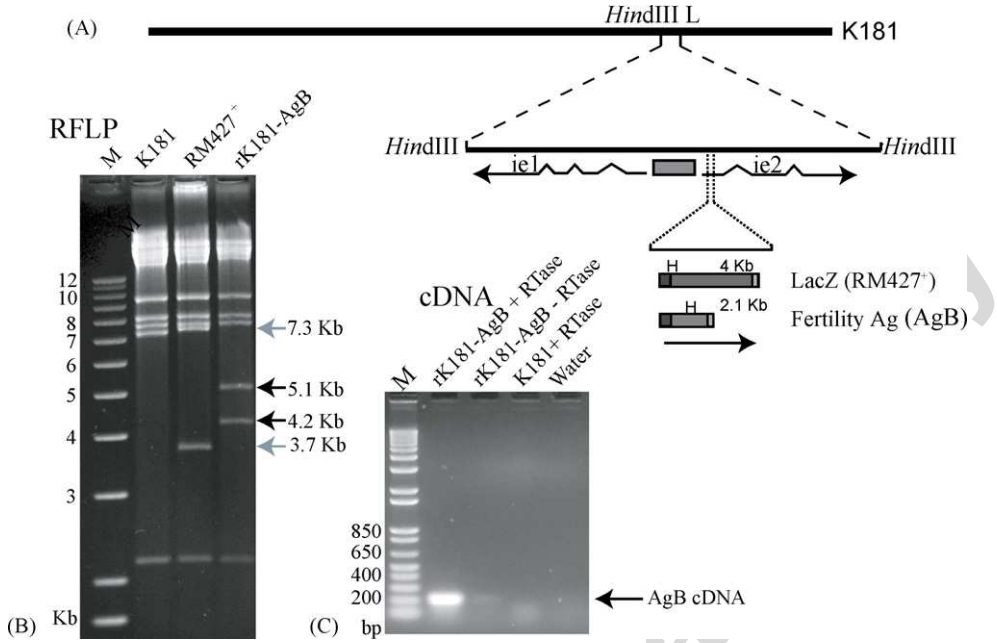

Fig. 1. Production of the recombinant vaccine strains of virus: (A) cloning strategy, rMCMV strains were produced by cloning the gene construct into the *ie2* gene. The intermediate RM427^{+} virus expressing the LacZ cassette allows for selection of "white" non-staining rMCMV plaques. The *ie2* gene is located within the *Hin*dIII L fragment. (B) RFLP analysis, RM427^{+} has an additional *Hin*dIII site (H), present with in the LacZ cassette compared to parental K181 strain MCMV. The 7161 bp *Hin*dIII L fragment in parental K181 is replaced by a 3756 and 7326 bp *Hin*dIII fragment in RM427^{+} (grey arrows). The rK181-AgB virus has an additional *Hin*dIII within the AgB polyepitope, *Hin*dIII digestion produces two novel fragments of 4178 and 5055 bp (black arrows). (C), The rK181-AgB virus expressed mRNA for the inserted construct shown as by the 220 bp RT-PCR product. Similar data was seen for the other rMCMVs (data not shown).

teristics the recombinant viruses were tested in vivo for their capacity to sterilise female mice.

3.3. Breeding studies

A total of five experiments were performed in testing the various recombinant viruses. Consequently there were a total of five independent breeding studies with the positive control virus, rK181-ZP3. Since there was no significant difference between the five sets of data, these data were pooled for presentation reasons. Similarly, negative control data sets for the diluent only or parental K181 were pooled when shown not to be significantly different from replicates (5 and 4 experiments, respectively). The majority of K181-infected or diluent-only treated mice remained fully fertile (Table 4).

Within the groups of mice infected with rMCMV expressing whole protein antigens, only those which received the control antigen, mZP3 (rK181-ZP3), or ubiquitin-tagged mZP3 (rK181-UbZP3) showed reduced fertility (Fig. 3A and Table 4). The rK181-ZP3 data is consistent with previous studies and is cumulative data from 28 mice in five independent experiments (Table 4). In this instance, rK181-ZP3 sterilised all the recipient female mice in 2/5 experiments (a

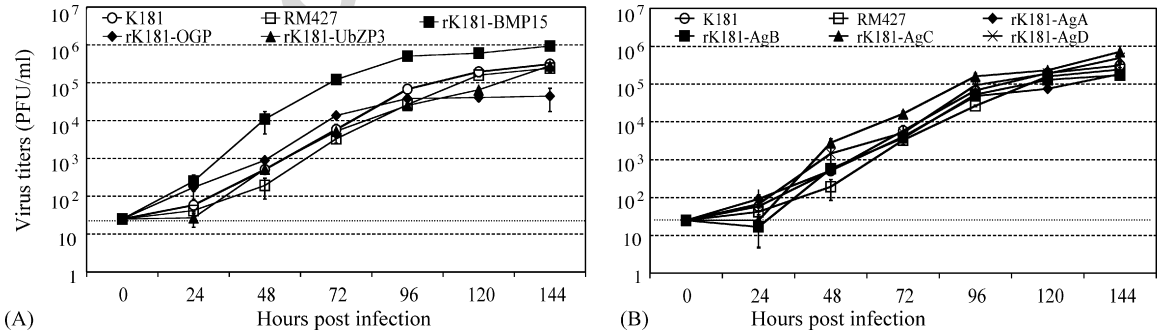

Fig. 2. Recombinant viruses demonstrated normal in vitro replication. All the rMCMVs produced and used in this study were assessed for their capacity for replication in cell culture. Parental K181 and rMCMVs were added to MEF at an MOI of 0.05 in a multi-step growth assay. (A) Viruses expressing protein antigens had similar replication kinetics to control RM427^{+} and parental K181 virus. (B) Similar data was seen with the recombinant viruses expressing polyepitope antigens with all displaying normal replication kinetics in vitro when compared to control viruses. The dotted line represents the limit of detection of the assay. Shown is the mean ± S.E.M. of virus titre.

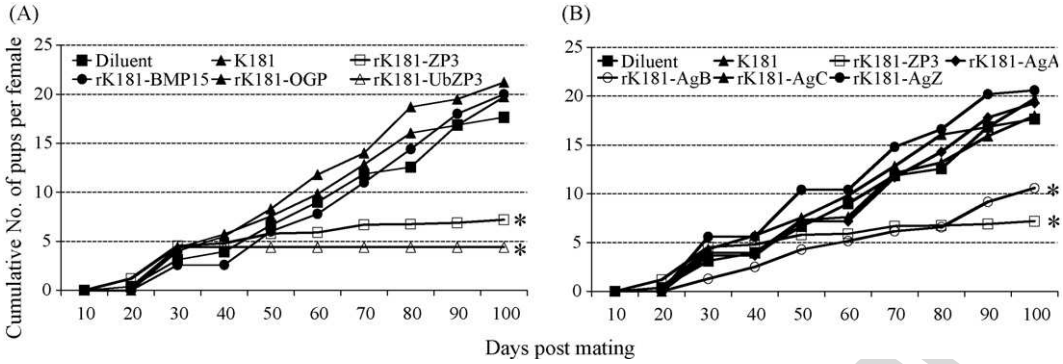

Fig. 3. Recombinant viruses differed in their ability to sterilise female BALB/c mice. Female BALB/c mice were inoculated i.p. with 2×10^4 PFU of tissue culture derived rMCMV constructs. In each experiment a positive control, consisting of mice inoculated with rK181-ZP3, was included. Negative control mice were inoculated with either parental K181 virus or diluent alone. The minimum number of females inoculated in any one treatment was five. Each female was paired with a single male immediately after inoculation. Shown in (A) rMCMVs expressing protein antigens and (B) rMCMVs expressing polyepitope antigens. Control data are the combination of all the individual studies. Data from rK181-BMP15, rK181-OGP, rK181-AgA and K181-AgZ were taken from a single study each. Data for rK181-AgC and rK181-AgB are compiled from of two independent experiments with $n = 16$ and 11 mice, respectively. Female mice that did not have a first litter, and were therefore not proven fertile at the start of the study, were excluded from this analysis. Asterisks denote significant difference (<0.05) between test virus and negative control mice.

total of 12 female mice). Most mice in the other three experiments had less than 2 litters, with only 3 out of a total of 16 remaining mice having more than three litters. Excluding the first litter, female mice infected with rK181-ZP3 had an average of 2.1 ± 0.7 pups over the 100-day period. This was significantly less than that seen in either negative control group. Diluent control treated mice had 14.3 ± 1.3 pups per female and K181 infected mice had 13.8 ± 1.3 pups per female mouse (Table 4).

Female BALB/c mice infected with rK181-UbZP3 had no litters after the first round of breeding and were completely infertile for the duration of the 100-day experiment (Fig. 3A and Table 4). All female and male mice in the rK181-UbZP3 treatment group were initially fully fertile, as all females had a first litter. This was significant reduction in breeding compared to the K181 infected or diluent treated mice (Fig. 3A and Table 4).

There was no demonstrable effect on the breeding success of female mice infected with rK181-BMP15 or rK181-OGP (Fig. 3A and Table 4). All mice in these groups were fertile for the full course of the experiment. Over the 100-day experiment, female mice infected with rK181-BMP15 had an average of 16.2 ± 1.6 pups per female mouse. Similar data was seen in female mice infected with rK181-OGP (14.8 ± 0.6 pups per female).

Variable results were obtained with the rMCMVs expressing epitope antigens (Fig. 3B and Table 4). Of those tested, only rK181-AgB demonstrated an effect on the fertility of female mice. Because sub-fertility was not seen in previous studies and because this data represented a decrease in breeding rather than infertility, this experiment was repeated. The rK181-AgB data in Fig. 3B and Table 4 are compilation of the data from two independent experiments. For 11 mice vaccinated with rK181-AgB there was an average of

Table 4
Compilation of data from all breeding trials

Virus inoculum	Total number inoculated	No. with first litter (%)	No. with >1 litter (%)	Mean no. of litters per female	Mean no. of pups per female	Mean no. of pups per female post first litter	Mean litter size after first litter
Diluent	24	23 (95.8)[a]	22 (95.6)	3.2 ± 0.2	18.0 ± 1.0	14.3 ± 1.3	6.2 ± 0.4
K181	19	18 (94.7)[a]	17 (94.4)	3.3 ± 0.2	19.7 ± 1.3	13.8 ± 1.3	6.1 ± 0.4
rK181-ZP3	28	24 (85.7)[a]	9 (37.5)	$1.6 \pm 0.2^*$	$7.2 \pm 0.9^*$	$2.1 \pm 0.7^*$	$3.6 \pm 0.7^*$
rK181-BMP15	5	5 (100)	5 (100)	3.4 ± 0.4	20.0 ± 1.0	16.2 ± 1.6	6.8 ± 1.1
rK181-UbZP3	5	5 (100)	0 (0)	$1.0 \pm 0^*$	$4.4 \pm 0.5^*$	0^*	n/a
rK181-OGP	6	6 (100)	6 (100)	3.7 ± 0.2	21.2 ± 1.2	14.8 ± 0.6	5.9 ± 0.7
rK181-AgA	6	6 (100)	6 (100)	3.8 ± 0.2	19.3 ± 2.1	15.7 ± 1.5	5.5 ± 1.0
rK181-AgB	11	11 (100)	11 (100)	2.7 ± 0.3	$10.6 \pm 1.4^*$	$6.9 \pm 1.3^*$	4.4 ± 0.6
rK181-AgC	16	16 (100)	16 (100)	3.5 ± 0.2	18.9 ± 1.7	14.6 ± 1.7	5.8 ± 0.4
rK181-AgD	5	5 (100)	5 (100)	3.8 ± 0.2	20.6 ± 2.0	15.0 ± 1.4	5.4 ± 1.1

Shown in a compilation of the data from all the breeding trials. Data from AgA and AgB are the compilation of two independent experiments. Data from K181-ZP3 is from 5 independent experiments and negative control data from K181 and diluent control treated mice from $n = 5$ and 4 experiments, respectively. Asterisks denote significant difference from K181 and diluent treated mice. Pooled data was analysed for significance by a one-way ANOVA with Tukey's honestly significant post hoc test.

[a] Female mice that were not confirmed fertile, by having a first litter, were excluded from subsequent analyses.

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6.9 ± 1.3 pups per female. This contrasts with the fully fertile controls (14.3 ± 1.3) (Table 4). This led to a significant reduction in the number of pups born to rK181-AgB infected mice compared to negative control mice (Fig. 3B). However, the number of litters produced by rK181-AgB infected mice was not significantly different to K181 infected mice, therefore the reduced number of pups per female was a result of reduced litter size rather than a reduction in the number of fertile animals (Table 4). These data indicate sub-fertility rather than infertility.

The other polyepitopes expressing viruses, rK181-AgA, rK181-AgC and rK181-AgZ had no effect on the fertility of female mice. The average number of pups per female for rK181-AgA ($n = 6$), rK181-AgC ($n = 16$) and rK181-AgZ ($n = 5$) infected mice for the 100-day experiment was 15.7 ± 1.5, 14.6 ± 1.7 and 15.0 ± 1.4, respectively, and was not significantly different to the negative control mice (Table 4). The results for the rK181-AgC virus are a compilation of two independent experiments.

3.4. Seroconversion

Although it was demonstrated that mRNA for all viral transgenes was expressed, it was not possible to assess seroconversion to all of the encoded antigens, as suitable antigen was not available. However, the mZP3 ELISA was used to test sera from all infected mice at the end of the 100-day study. Both the percentage of mice producing antibody to mZP3 and the average titre of antibody in the serum of these mice was assessed and are shown for IgG1 and IgG2a (Fig. 4). Mice infected with the rK181-UbZP3 virus demonstrated a seroconversion rate of 100% by the end of the 100-day experiment. This was seen with both IgG1 and IgG2a isotypes (Fig. 4C and D). In contrast only 57% of female mice infected with rK181-ZP3 were mZP3 seropositive after 100 days. However, the titre of the mZP3 antibodies in the sera of rK181-ZP3 and rK181-UbZP3 infected mice that were ZP3 seropositive was similar (Fig. 4A and B). The serum from the rK181-ZP3 infected mice was taken from two independent experiments ($n = 7$ mice).

As expected, female BALB/c mice were seronegative for mZP3 antibody when inoculated with rK181-BMP15 or rK181-OGP. This was seen for both IgG2a and IgG1 isotypes, although two female mice infected with rK181-OGP produced low titre cross-reactive IgG1 antibody to the mZP3 ELISA antigen (Fig. 4C and D).

The mZP3 seropositivity was variable with the polyepitope expressing viruses. There was a trend to increased production of mZP3 antibody in the rK181-AgB infected mice compared to mice infected with AgA, AgC or AgZ expressing

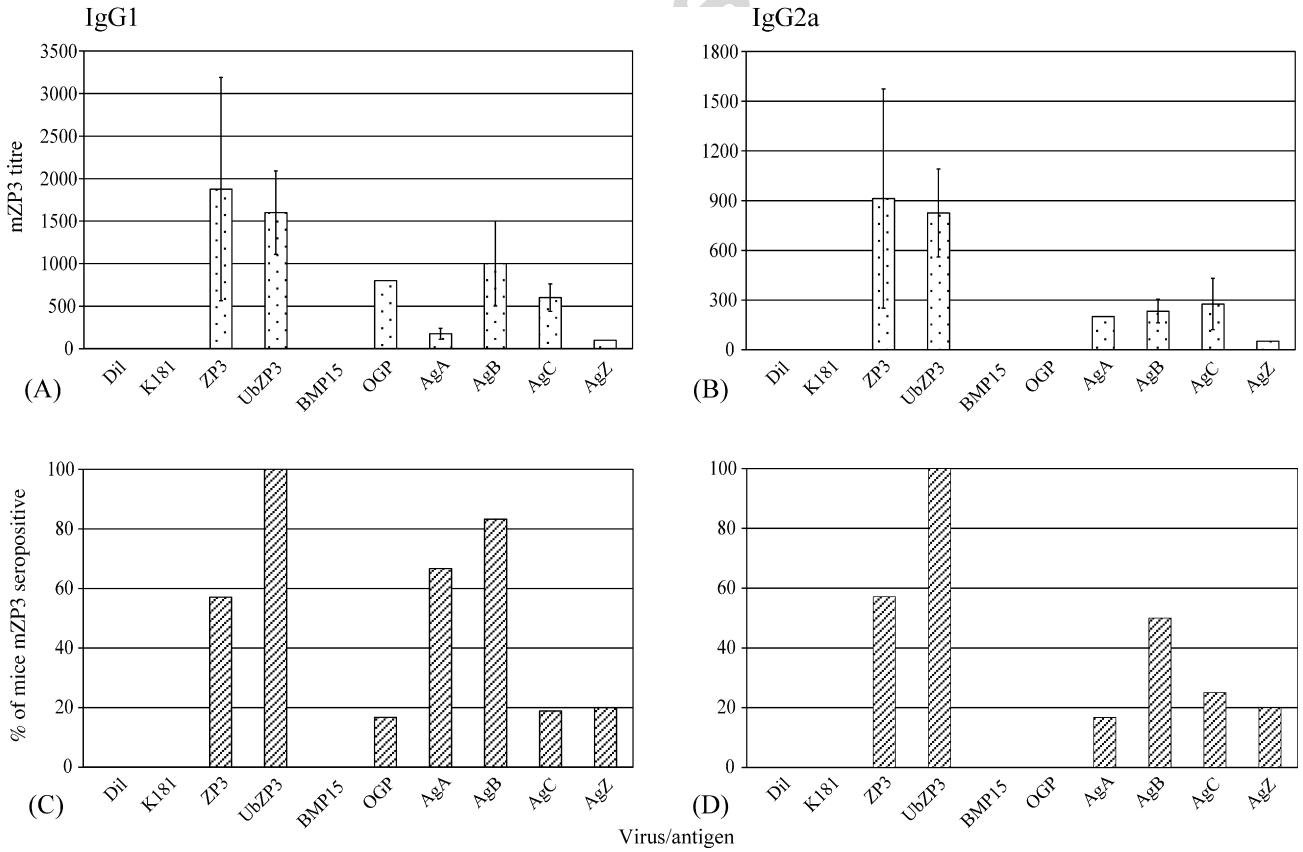

Fig. 4. Production of antibody to mZP3 in mice infected with recombinant viruses. Serum was collected from female mice at the end of each 100-day breeding study and the presence of anti-mZP3 antibody was assessed by ELISA. (A) mZP3 IgG1 titre, (C) percentage of mice positive for IgG1 mZP3 antibodies. (B) mZP3 IgG2a titre, (D) percentage of mice positive for Ig2a mZP3 antibodies.

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viruses. However, the mZP3 antibody titres were low in mice infected with rMCMV-polyepitope viruses (Fig. 4A–D).

4. Discussion

We have screened a range of self-protein antigens and synthetic polyepitope antigens for potential use in an immunocontraceptive vaccine using the MCMV vector. In female BALB/c mice, only those viruses expressing mZP3 antigens had any effect on fertility. The full-length mZP3 (in 2/5 experiments) and full-length ubiquitin-tagged mZP3 induced complete infertility in 100% of infected mice. Of those viruses expressing polyepitope antigens only the virus expressing AgB had any effect on female mouse fertility. The rK181-AgB virus induced sub-fertility rather than infertility. AgB contains the contraceptive epitopes SP56, ZP3-T, ZP3-B and ZP1 and the helper epitopes BRN-T, HA-T. Viruses expressing BMP15 or OGP had no effect on the fertility of mice, nor did viruses expressing the polyepitope antigens A, C or Z.

Sterilisation of female BALB/c mice by rK181-ZP3 was anticipated as we have shown the efficacy of this approach in previous studies [11,12]. Accordingly, this vaccine was used in this study as a positive control. The N-terminal ubiquitin-tagged mZP3 has not previously been assessed. Ubiquitination targets proteins for degradation via the 26S proteosome, and increases antigen presentation via the MHC class I pathway [42]. In vaccine studies, ubiquitination has been shown to enhance both the degradation of tagged antigens in the proteosome and the CD8 cytotoxic T cell response, whilst reducing antibody responses [28,29,43]. Ubiquitinated antigens have been shown to enhance protective responses to expressed antigens in several model systems [29,43–46].

The use of ubiquitin-tagged antigens has been predominantly studied with DNA vaccines. We report here the use of a virally expressed, ubiquitin-tagged immunocontraceptive and report that it is equally as successful as the non-tagged mZP3 protein. In this study the mZP3 protein was N-terminally tagged with the ubiquitin monomer as has been described for other antigens [29]. This construct was designed with a G26 to A26 amino acid substitution to prevent cleavage and allow for polyubiqitination [29].

In this study the MHC class I restricted CD8 T cell response was not assessed. However, contrary to expectations the antibody response to mZP3 was raised. This was reflected by the fact that at 100 days post infection 100% of mice infected with rK181-UbZP3 remained mZP3 seropositive. This contrasts with 57% seropositivity in mice infected with the non-ubiquitin-tagged mZP3 expressing virus (rK181-ZP3). This was seen with both IgG1 and IgG2a isotypes. However, of those seropositive, the antibody titres were similar for rK181-ZP3 and rK181-UbZP3 infected female BALB/c mice. The low antibody levels seen in other ubiquitin-tagged antigen studies could be due to the different systems used. Early studies using DNA vaccines postulated that the lack of an antibody response was due to rapid degradation of the antigen [29]. The levels of antigen expressed by a DNA vaccine compared to those produced by a virus are likely to be considerably less. This increased antigen expression may allow sufficient protein expression for both CTL and antibody responses. In addition, the use of a virus, rather than a plasmid, to express the antigen is likely to stimulate more host immune response pathways.

The research reported here indicates that rK181-UbZP3 may be more efficient than rK181-ZP3 in the induction of reproductive sterility. However, as the rK181-ZP3 itself induces infertility at a rate approaching 100%, and in two of five experiments did reach 100%, any increase in vaccine efficacy is difficult to measure. Future studies will focus on the $CD8^+$ T cell responses to the ubiquitin-tagged mZP3 and the potential for this vaccine to be used in inbred strains of mice which are refractory to the induction of infertility by rK181-ZP3.

Both BMP15 and OGP when expressed by MCMV failed to induce infertility in female mice. BMP15, otherwise known as GDF9b, is a member of the transforming growth factor-β (TGFβ) superfamily. BMP15 is expressed almost exclusively in the oocyte and is first evident at the primary follicle stage, expression continues through to ovulation [25]. BMP15 has a range of functions that could be expected to affect female mouse fertility (reviewed in ref. [26]). OGP is mostly associated with the zona pellucida or perivitelline space in mammalian oviductal oocytes and early cleavage stage embryos [27]. This suggests it may have a role in fertilisation or early embryonic development [27,47–49], making it a candidate for an immunocontraceptive vaccine. However, both rK181-BMP15 and rK181-OGP viruses failed to affect the fertility of female mice. It is noteworthy that the protein antigen, mZP3, that sterilises mice in this system is essential for female mouse fertility [23,24]. In contrast, both BMP15 and OGP knockout mice remain fertile [50,51]. It should be noted however, that whilst mRNA for both BMP15 and OGP could be detected in cells infected with rK181-BMP15 and rK181-OGP viruses, the translation of protein from these transcripts was not assessed. Hence, it remains possible that rK181-BMP15 and rK181-OGP viruses failed to sterilise mice due to a lack of protein translation or due to low protein production.

Only rK181-AgB, of the polyepitope vaccines, affected the breeding of female mice. Whilst this was seen only as a reduction in fertility, or sub-fertility, the effect was long lasting. These results have considerable importance for the ultimate aim of these studies, which is to produce a fully disseminating viral vectored immunocontraceptive that will control the fertility of wild mice in plague prone regions in Australia. The requirement for such a vaccine would be two-fold. The virus should sterilise or significantly reduce the fertility of most mice and the effect must be species specific [32]. MCMV was selected as the vaccine vector for these studies as it is a natural pathogen of mice and is strictly species specific. However, the inclusion of a mouse specific antigen would enhance the safety of this approach. In this regard, the individual epitopes used in each synthetic polyepitope

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were chosen for their putative mouse or rodent specific nature [30,31]. Hence, this study has demonstrated that it is possible to use a mouse specific virus to deliver mouse specific epitopes and induce prolonged sub-fertility in recipient animals. Further studies will focus on enhancing the efficacy of AgB, such as the inclusion of an ubiquitin tag, or other mouse specific polyepitopes.

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References

[1] Andino R, Silvera D, Suggett SD, et al. Engineering poliovirus as a vaccine vector for the expression of diverse antigens. Science 1994;265(5177):1448–51.
[2] Mandl S, Sigal LJ, Rock KL, Andino R. Poliovirus vaccine vectors elicit antigen-specific cytotoxic T cells and protect mice against lethal challenge with malignant melanoma cells expressing a model antigen. Proc Natl Acad Sci USA 1998;95(14):8216–21.
[3] Ramirez JC, Gherardi MM, Esteban M. Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine. J Virol 2000;74(2):923–33.
[4] Berencsi K, Gyulai Z, Gonczol E, et al. A canarypox vector-expressing cytomegalovirus (CMV) phosphoprotein 65 induces long-lasting cytotoxic T cell responses in human CMV-seronegative subjects. J Infect Dis 2001;183(8):1171–9.
[5] Marshall JL, Hawkins MJ, Tsang KY, et al. Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. J Clin Oncol 1999;17(1):332–7.
[6] McGettigan JP, Pomerantz RJ, Siler CA, et al. Second-generation rabies virus-based vaccine vectors expressing human immunodeficiency virus type 1 gag have greatly reduced pathogenicity but are highly immunogenic. J Virol 2003;77(1):237–44.
[7] Shiver JW, Fu TM, Chen L, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 2002;415(6869):331–5.
[8] Brockman MA, Knipe DM. Herpes simplex virus vectors elicit durable immune responses in the presence of preexisting host immunity. J Virol 2002;76(8):3678–87.
[9] Murphy CG, Lucas WT, Means RE, et al. Vaccine protection against simian immunodeficiency virus by recombinant strains of herpes simplex virus. J Virol 2000;74(17):7745–54.
[10] Jackson RJ, Maguire DJ, Hinds LA, Ramshaw IA. Infertility in mice induced by a recombinant ectromelia virus expressing mouse zona pellucida glycoprotein 3. Biol Reprod 1998;58(1):152–9.
[11] Lloyd ML, Shellam GR, Papadimitriou JM, Lawson MA. Immunocontraception is induced in BALB/c mice inoculated with murine cytomegalovirus expressing mouse zona pellucida 3. Biol Reprod 2003;68(6):2024–32.
[12] Redwood AJ, Messerle M, Harvey NL, et al. Use of a murine cytomegalovirus, K181-derived, bacterial artificial chromosome as a vaccine vector for immunocontraception. J Virol 2005;79(5):2998–3008.
[13] McKenzie SM, McLaughlin EA, Perkins HD, et al. The immunocontraceptive effects on female rabbits infected with recombinant myxoma virus expressing rabbit ZP2 or ZP3. Biol Reprod 2006;74(3):511–21.
[14] Rawlinson WD, Farrell HE, Barrell BG. Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol 1996;70(12):8833–49.
[15] Sweet C. The pathogenicity of cytomegalovirus. FEMS Microbiol Rev 1999;23(4):457–82.
[16] Khan N, Shariff N, Cobbold M, et al. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol 2002;169(4):1984–92.
[17] Karrer U, Sierro S, Wagner M, et al. Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. J Immunol 2003;170(4):2022–9.
[18] Alford CA, Britt WJ, Cytomegalovirus. In: Fields BN, Knipe DM, editors. Fields virology, vol. 2. New York: Raven Press; 1990. p. 1981–2010.
[19] Tischer BK, Schumacher D, Beer M, et al. A DNA vaccine containing an infectious Marek's disease virus genome can confer protection against tumorigenic Marek's disease in chickens. J Gen Virol 2002;83(Pt 10):2367–76.
[20] Suter M, Lew AM, Grob P, et al. BAC-VAC, a novel generation of (DNA) vaccines: a bacterial artificial chromosome (BAC) containing a replication-competent, packaging-defective virus genome induces protective immunity against herpes simplex virus 1. Proc Natl Acad Sci USA 1999;96(22):12697–702.
[21] Cicin-Sain L, Brune W, Bubic I, Jonjic S, Koszinowski UH. Vaccination of mice with bacteria carrying a cloned herpesvirus genome reconstituted in vivo. J Virol 2003;77(15):8249–55.
[22] Davidson A, Diamond B. Autoimmune diseases. N Engl J Med 2001;345(5):340–50.
[23] Liu C, Litscher ES, Mortillo S, et al. Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. Proc Natl Acad Sci USA 1996;93(11):5431–6.
[24] Rankin T, Familari M, Lee E, et al. Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. Development 1996;122(9):2903–10.
[25] Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM. The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 1998;12(12):1809–17.
[26] Juengel JL, McNatty KP. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. Hum Reprod Update 2005;11(2):143–60.
[27] Boice ML, McCarthy TJ, Mavrogianis PA, Fazlebas AT, Verhage HG. Localization of oviductal glycoproteins within the zona pellucida and perivitelline space of ovulated ova and early embryos in baboons (Papio anubis). Biol Reprod 1990;43(2):340–6.
[28] Andersson HA, Barry MA. Maximizing antigen targeting to the proteasome for gene-based vaccines. Mol Ther 2004;10(3):432–46.
[29] Rodriguez F, Zhang J, Whitton JL. DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. J Virol 1997;71(11):8497–503.
[30] Hardy CM, Clydesdale G, Mobbs KJ. Development of mouse-specific contraceptive vaccines: infertility in mice immunized with peptide and polyepitope antigens. Reproduction 2004;128(4):395–407.
[31] Hardy CM, Pekin J, ten Have J. Mouse-specific immunocontraceptive polyepitope vaccines. Reprod Suppl 2002;60:19–30.
[32] Shellam GR. The potential of murine cytomegalovirus as a viral vector for immunocontraception. Reprod Fertil Dev 1994;6(3):401–9.
[33] Cardin RD, Abenes GB, Stoddart CA, Mocarski ES. Murine cytomegalovirus IE2, an activator of gene expression, is dispensable for growth and latency in mice. Virology 1995;209(1):236–41.
[34] Xu J, Dallas PB, Lyons PA, Shellam GR, Scalzo AA. Identification of the glycoprotein H gene of murine cytomegalovirus. J Gen Virol 1992;73(Pt 7):1849–54.

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[35] Davis-Poynter NJ, Lynch DM, Vally H, et al. Identification and characterization of a G protein-coupled receptor homolog encoded by murine cytomegalovirus. J Virol 1997;71(2):1521–9.
[36] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162(1):156–9.
[37] Finch JS, Bonham K, Krieg P, Bowden GT. Murine polyubiquitin mRNA sequence. Nucleic Acids Res 1990;18(7):1907.
[38] Teasdale RD, D'Agostaro G, Gleeson PA. The signal for Golgi retention of bovine beta 1,4-galactosyltransferase is in the transmembrane domain. J Biol Chem 1992;267(18):25.
[39] Stearne PA, Pietersz GA, Goding JW. cDNA cloning of the murine transferrin receptor: sequence of trans-membrane and adjacent regions. J Immunol 1985;134(5):3474–9.
[40] Karupiah G, Sacks TE, Klinman DM, et al. Murine cytomegalovirus infection-induced polyclonal B cell activation is independent of CD4+ T cells and CD40. Virology 1998;240(1):12–26.
[41] Price P, Olver SD, Gibbons AE, Shellam GR. B-cell activation following murine cytomegalovirus infection: implications for autoimmunity. Immunology 1993;78(1):14–21.
[42] Grant EP, Michalek MT, Goldberg AL, Rock KL. Rate of antigen degradation by the ubiquitin-proteasome pathway influences MHC class I presentation. J Immunol 1995;155(8):3750–8.
[43] Liu WJ, Zhao KN, Gao FG, Leggatt GR, Fernando GJ, Frazer IH. Polynucleotide viral vaccines: codon optimisation and ubiquitin conjugation enhances prophylactic and therapeutic efficacy. Vaccine 2001;20(5–6):862–9.
[44] Niethammer AG, Xiang R, Ruehlmann JM, et al. Targeted interleukin 2 therapy enhances protective immunity induced by an autologous oral DNA vaccine against murine melanoma. Cancer Res 2001;61(16): 6178–84.
[45] Sykes KF, Johnston SA. Genetic live vaccines mimic the antigenicity but not pathogenicity of live viruses. DNA Cell Biol 1999;18(7): 521–31.
[46] Tellam J, Sherritt M, Thomson S, et al. Targeting of EBNA1 for rapid intracellular degradation overrides the inhibitory effects of the Gly-Ala repeat domain and restores CD8+ T cell recognition. J Biol Chem 2001;276(36):33353–60.
[47] Boatman DE, Magnoni GE. Identification of a sperm penetration factor in the oviduct of the golden hamster. Biol Reprod 1995;52(1):199–207.
[48] O'Day-Bowman MB, Mavrogianis PA, Reuter LM, Johnson DE, Fazleabas AT, Verhage HG. Association of oviduct-specific glycoproteins with human and baboon (Papio anubis) ovarian oocytes and enhancement of human sperm binding to human hemizonae following in vitro incubation. Biol Reprod 1996;54(1):60–9.
[49] Schmidt A, Mavrogianis PA, O'Day-Bowman MB, Jaffe RC, Verhage HG. Characterization of antibodies generated against a conserved portion of oviductal glycoprotein (OGP) and endogenous hamster OGP and their ability to decrease sperm binding to the zona pellucida in vitro. Am J Reprod Immunol (Copenhagen) 1997;38(6):377–83.
[50] Yan C, Wang P, DeMayo J, et al. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 2001;15(6):854–66.
[51] Araki Y, Nohara M, Yoshida-Komiya H, et al. Effect of a null mutation of the oviduct-specific glycoprotein gene on mouse fertilization. Biochem J 2003;374(Pt 2):551–7.

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