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Immunocontraceptive Effects on Female Rabbits Infected with Recombinant Myxoma Virus Expressing Rabbit ZP2 or ZP3

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ABSTRACT

Recombinant myxoma viruses expressing rabbit zona pellucida 2 (rZP2) or rabbit zona pellucida 3 (rZP3) glycoproteins were constructed and tested in domestic rabbits to assess their potential to induce autoimmune infertility. The recombinant virus expressing rZP2 had no effect on fertility or ovarian histology, despite all animals developing antibodies against the rZP2 antigen. However, recombinant viruses expressing rZP3 induced infertility in 70% of animals at the first breeding. Serum antibodies were relatively short-lived, but antibody was bound to zona pellucida of all rabbits from Day 10 onward. There was no obvious correlation between infertility and rZP3 antibody titer. There was a transient inflammatory response in the ovaries of rZP3-immunized rabbits at Day 15 but no T-cell response to rZP3 could be detected at any time. Dysfunctional follicular formation was present in ovaries from rabbits infected with rZP3-expressing viruses 15–40 days postinfection but this had disappeared at later time points. A recombinant myxoma virus expressing a modified rZP3 antigen with the C-terminal hydrophobic putative anchor sequence deleted was also tested. This virus did not induce either infertility or an antibody response against the zona pellucida. Thus, the context of antigen presentation was crucial for an autoimmune response.

autoimmunity, female reproductive tract, immunology, immunocontraception, myxoma virus, rabbits, zona pellucida 3

INTRODUCTION

Immunization with antigens derived from the zona pellucida is able to induce autoimmune infertility in animals ranging from mice and rabbits to wild deer, horses, and elephants [1–5]. However, these trials have used direct injection of zona pellucida antigens. Such a strategy is not feasible for fertility control of widely distributed vertebrate pests such as the European rabbit (*Oryctolagus cuniculus*) in Australia. Therefore, it has been suggested that a genetically engineered myxoma virus could be developed as a vector for delivering immunocontraceptive antigens to rabbits [6]. Such a strategy requires identification of a suitable antigen that can be delivered in a recombinant virus and induce autoimmune infertility in at least 60%–80% of female rabbits in a population [7]. In previous studies, it has been demonstrated that recombinant myxoma viruses can induce a strong immune response to a virally-expressed foreign antigen [8, 9]. In addition, it has been demonstrated that direct injection of the alloantigen rabbit ZP1 (rZP1) as a recombinant protein induced autoimmune infertility. However, when rZP1 was delivered by a recombinant myxoma virus, although immune tolerance was overcome, there was no significant infertility [10, 11].

The zona pellucida is a complex extracellular matrix that surrounds the oocyte. In the rabbit, it is composed of three major sulphated glycoproteins that have been termed ZPA, ZPB and ZPC [12]. These equate to the mouse/human ZP2, ZP1, and ZP3, respectively. For the rabbit the predicted molecular masses are 75 kDa, 55 kDa, and 45 kDa [13]. The nomenclature of these genes and their encoded proteins is complicated, with unifying proposals made by Prasad et al. (2000) [14] and Spargo and Hope (2003) [15].

In rabbits, rZP1 is the major component of the zona pellucida, and both rZP1 and rZP3 are involved in sperm binding [16]. The roles of the zona pellucida in oocyte development and in attachment and binding of sperm are critical for reproductive success (reviewed in [14]). All three zona proteins are heavily glycosylated with both O- and N-linked carbohydrate chains. The oligosaccharide component of the zona pellucida is essential for sperm binding and fertilization [17]. The antigenic importance of this carbohydrate component in developing autoimmune responses to recombinant zona pellucida proteins appears to vary with the species immunized, but in some cases may be important in inducing ovarian autoimmune disease following immunization [18–23].

Immunization of rabbits with whole porcine zona pellucida has been shown to induce a strong autoimmune response and cause infertility, endocrine dysfunction, and ovarian pathology [3, 24, 25]. However, immunization with whole rabbit zona pellucida did not induce infertility [3]. In contrast, mammalian-cell-expressed rZP1 was an effective contraceptive antigen in rabbits when delivered with adjuvant with boosting, resulting in sustained infertility in 70% of female domestic rabbits [10].

In mice, delivery of the murine ZP3 protein by either a recombinant mousepox virus [26] or a recombinant murine cytomegalovirus [27] induced autoimmune responses to ZPC,

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leading to long-term infertility. Induction of a similar autoimmune response in rabbits by a recombinant myxoma virus has proved more difficult. When rZP1 was delivered by a recombinant myxoma virus, although rabbits developed an antibody response to rZP1, there was no effect on fertility. If this response was boosted with two immunizations of rZP1 protein, then high antibody titers, delayed-type hypersensitivity (DTH) responses, and infertility were induced. However, boosting with protein would not be practical as a rabbit control method. Furthermore, boosting with recombinant myxoma virus was not effective because of the long-lived protective immunity to myxoma virus induced by the first infection [10]. Therefore, it was considered necessary to look for more effective antigens to use with a recombinant myxoma virus.

MATERIALS AND METHODS

Construction of Recombinant Myxoma Viruses Using Transient Dominant Selection

Recombinant virus construction and testing were done under license from the Commonwealth of Australia Office of the Gene Technology Regulator.

Plasmid construction. A transient dominant selection transfer vector, pGGG-L (Fig. 1), was constructed to prepare recombinant myxoma viruses expressing rZP2 or rZP3. The *rZP2* cDNA clone available was missing the 5′ region encoding the amino-terminal signal sequence [28]. To reconstruct the *rZP2* sequence, the 5′ region from *rZP1* encoding the signal sequence was used to create a *rZP1-rZP2* fusion construct, thereby theoretically reconstructing a fully functional *rZP2* sequence. A *Cla*I/*Bcl*I restriction fragment that contained the *TK* gene (*M061R*), a synthetic vaccinia virus late promoter, and the cDNA region of *rZP1* encoding the leader sequence was removed from p*URTK11-ZPB* [10]. This DNA fragment was ligated in-frame with the 5′ end of *rZP2* at an introduced *Bam*HI site. The entire DNA fragment was then ligated into a *Cla*I/*Sal*I restriction site of pGGG-L to make pGGG-L-*rZP2*.

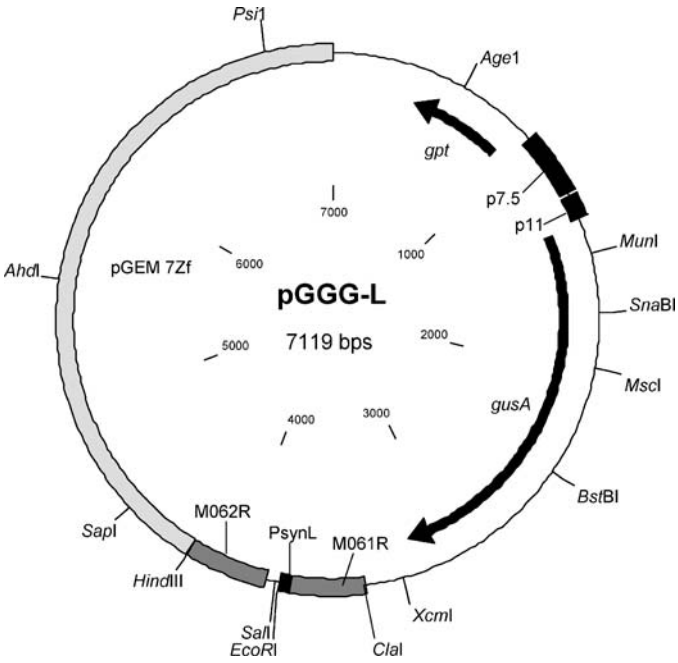

FIG. 1. Plasmid constructed for transient dominant selection. The antigen gene is inserted into an *Eco*RI/*Sal*I restriction site and is under the transcriptional regulation of a synthetic late promoter. The antigen gene is flanked by the *M061R* and *M062R* genes of myxoma virus for homologous recombination. The transient dominant selection markers include the *gpt* gene, which is under the transcriptional control of the p7.5 vaccinia virus early/late promoter element, and the *gusA* (*β-glucuronidase*) gene under the transcriptional control of the poxvirus p11 promoter.

The *rZP3* cDNA clone was missing a small 5′ region encoding 4 amino acids (MGLS) based on sequence homology analysis of other species [12]. The missing 5′ sequence was reconstructed by the insertion of a synthetic DNA linker made up of complementary oligonucleotides (5′-AATTCCGCCAC-CATGGGGCTGAGCTACGGGCTCTTCG-3′, and 5′-AAACGAA-GAGCCCGTAGCTCAGCCCCATGGTGGCGG-3′). The complementary synthetic oligonucleotides include a 5′ Kozak sequence (underlined) and 5′ *Eco*RI overhang, and a 5′ *Ear*I underhang sequence for ligation at the *Ear*I restriction site of *rZP3*. The full *rZP3* sequence was then ligated into the *Eco*RI/*Sal*I restriction site of pGGG-L to make pGGG-L-*rZP3*. Attempts to select recombinant viruses with this construct were unsuccessful, possibly because of toxicity of the high level expression of rZP3 protein by the recombinant virus. To reduce expression of rZP3, site directed mutagenesis using the GeneEditor Kit (Promega) was used to change the TAAAT motif of the synthetic late promoter to TAAAA to decrease the strength of the promoter [29], thereby reducing expression of rZP3 protein. This construct was named pGGG-L(A)*rZP3*.

Selection of recombinant viruses. Recombinant myxoma viruses were constructed using the myxoma virus strain Uriarra (Ur), which was derived from Uriarra 2/53-1 by plaque purification [30]. This strain of virus was used to infect RK13 cell monolayers (a rabbit kidney cell line ATCC no.CCL-37) at a multiplicity of infection (moi) of 0.1 plaque-forming units (pfu) per cell. One hour after infection the cells were transfected with either pGGG-L-*rZP2* or pGGG-L(A)-*rZP3* using Qiagen Effectene transfection reagent (Qiagen) as recommended by the manufacturer. The cells were grown without selection in MEM (Gibco BRL) with 8% (v/v) newborn calf serum (Invitrogen) for 1 wk at 35°C, 5%CO_2/95% air. Infected cell lysate was prepared by freeze/thawing and then sonicating for 10 sec three times at a constant cycle in a Branson Sonifier 250. The cell lysate was then used to infect confluent monolayers of RK13 cells. After 24 h, the medium was replaced with selection medium (MEM, 1% [v/v] newborn calf serum, 5 μg/ml mycophenolic acid, 250 μg/ml xanthine, 15 μg/ml hypoxanthine, 2 μg/ml aminopterin, and 10 μg/ml thymidine). The infected culture was grown for 1 wk to allow the virus to amplify under selection pressure. Expression of the *gusA* gene was detected by blue color after the addition of 1% (w/v) Seaplaque CTG (BMA) agarose overlay containing 0.2 mg/ml of X-gluc (5′Bromo-4-Chloro-3-Indolyl-beta-D-Glucuronide) [31, 32]. Virus resulting from this amplification was then plaque-purified twice under selection. A further two rounds of plaque purification were then performed without mycophenolic acid or X-gluc selection.

PCR screening to identify recombinant viruses. Individual plaques were screened for the presence of wild-type Ur virus or the recombinant Ur-rZP2 or Ur-rZP3 by PCR. Oligonucleotides GTCATGAACAACAGTGCTGC and AGGCTCAGAGTTTGGGCTCC were used to amplify a 230-bp fragment internal to the *rZP2* cDNA. Oligonucleotides GATCGCTGCGTGGC-CACCCC and CGATGAGGTCACAGTCACCG were used to amplify a 350-bp fragment internal to the *rZP3* cDNA. To detect both contaminating wild-type virus and the full-length cDNA of either *rZP2* or *rZP3*, oligonucleotides annealing to regions within the MO61R gene (GATCGAAA-TAATAGGAGGTAGCGAC) and M062R (AACTCCACCGACTCGTCGT-TA) DNA sequences were used in the PCR reaction. PCR was performed, using *Taq* DNA polymerase (Qiagen) as follows: 30 cycles of 94°C for 30 s, 52°C for 1 min, 72°C for 30 s for small fragments (500 bp or less), and 1 min 30 s for larger fragments (up to 1.5 kb).

Construction of recombinant myxoma viruses incorporating gpt/gus *as a selection marker.* Two further recombinant myxoma viruses were constructed expressing either rZP3 protein or a modified rZP3 protein in which the 3′ region of the cDNA, encoding a putative membrane anchor sequence, was deleted to encode an anchorless rZP3 (rZP3-anc-gpt/gus). These viruses were constructed such that the selection gene *gpt*/*gusA* was permanently inserted into the genome of the recombinant virus. To construct rZP3-anc-gpt/gus the DNA encoding *rZP3-anc* was amplified by PCR using *Pfu* polymerase using the primers AATACCATGGCTGCACTTCTGCTCC and TATAGTC-GACTCACGCCTGCGCAGCAGAGGCC and subsequently subcloned into a standard plasmid vector. This subclone was sequenced to confirm the correct sequence before transferring into the *Eco*RI/*Sal*I restriction site of the myxoma transfer vector pUrFect.

The pUrFect plasmid vector was designed to enable efficient transfection of various genes into the Uriarra strain of myxoma virus. Using the pT7/*gpt-gus* plasmid [33] as a base, approximately 350 bp of myxoma virus sequence from the *TK* gene (*M061R*) and *MJ2a* gene (*M062R*) were incorporated either side of the *gpt*/*gusA* fusion sequence to facilitate homologous recombination with viral genomic sequences. Unique *Eco*RI and *Sal*I sites were positioned between the *gpt*/*gusA* and *MJ2a* sequences for insertion of additional sequences to be transfected into myxoma virus. In the final constructs, the transcription of *gpt*/*gusA* was driven by the synthetic early/late promoter [33], whereas transcription from *rZP3* or *rZP3-anc* were driven by the poxvirus p11 late promoter. The

subsequent recombinant viruses were designated Ur-rZP3-gpt/gus and Ur-rZP3-anc-gpt/gus.

Transfection and selection conditions in RK13 cells were as described above. Recombinant viruses were selected by repeated plaque purification and amplification until no contaminating wild-type virus could be demonstrated by PCR using specific primer pairs. Stocks of each recombinant virus were prepared in RK13 cells and titrated by plaque assay on Vero cell monolayers.

Expression and Purification of FLAG-rZP2 and FLAG-rZP3

Rabbit ZP2 and rZP3 were expressed as soluble FLAG fusion proteins using the vaccinia virus T7 (VV-T7) expression system [34, 35]. The *rZP2* and *rZP3* cDNAs encoding the mature protein were subcloned into the multiple cloning site of the pFLAG-CMV-1 expression vector (Sigma) downstream and in frame with a *preprotrypsin-FLAG* sequence. The *preprotrypsin-FLAG-ZP* construct was then transferred from the pFLAG-CMV-1 vector to a vaccinia virus transfer vector pTM1p11*gus*. This vector is based on pTM1 [35, 36]. The vector was modified to include the color selection *E. coli gusA* gene under the control of the p11 poxvirus promoter. The *preprotrypsin-FLAG-rZP2* or *preprotrypsin-FLAG-rZP3* cDNAs were inserted into the *Nco*I/*Sal*I site of pTMp11*gus*. The *rZP2* region encoding the putative amino terminal membrane anchor sequence was removed to facilitate construction of the final vector pTMp11*gus-pptFLAGZP2*. Recombinant vaccinia viruses were produced using published methods [37].

The FLAG-rZP2 and FLAG-rZP3 fusion proteins were expressed in CV-1 cells in serum-free MEM using the recombinant vaccinia virus/T7 RNA polymerase hybrid system [37]. Infected cell supernatant was concentrated 10-fold by freeze-drying and resuspending in PBS pH 7.4, 50 mM N-acetyl-glucosamine, 0.1% (w/v) TX-100, 10% (w/v) glycerol. The FLAG-rZP2 and FLAG-rZP3 were affinity-purified using a 1-ml Anti-Flag M2 affinity gel (Sigma) column. The proteins were passed over the column in the same buffer used to resuspend the proteins and eluted with 0.1 M glycine pH 3.5, 0.1% (w/v) TX-100, 5% (w/v) glycerol, and 50 mM *N*-acetyl-glucosamine. Eluents were neutralized with 1 M Tris, pH 8. Immunoblotting using antiflag monoclonal antibody demonstrated that proteins of the appropriate size were expressed.

Immunodetection of rZP2 and rZP3

Zona proteins expressed by recombinant myxoma viruses were detected by immunoblotting as follows. SDS-soluble proteins prepared from RK13 cells infected with recombinant virus were separated by discontinuous SDS-PAGE, then transferred to PVDF membranes using standard procedures [38]. Zona proteins were detected by immunolabeling with polyclonal rabbit sera raised against heat solubilized porcine zona (1:1000) followed by a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody.

To examine the cell surface expression of rZP3 expressed by Ur-rZP3-gpt/gus and Ur-rZP3-anc-gpt/gus, RK13 cells were infected at a multiplicity of infection (moi) of 1, and 16 h after infection the monolayers were washed and fixed with either 1:1 (v/v) methanol/acetone to permeabilize the cells or 4% formaldehyde to maintain membrane integrity. Expression of rZP3 in the cell or on the membrane was detected using polyclonal rabbit anti-porcine whole zona antiserum (1/400 dilution) followed by detection with anti-rabbit FITC and fluorescent microscopy.

Detection of Antibody Responses by ELISA

Wells of a 96-well plate (Greiner microlon 600) were coated with affinity purified FLAG-rZP3 antigen in carbonate buffer (pH 9.6) at a concentration that was preoptimized by antigen titration to give the highest optical density at the lowest dilution of control antibody (monoclonal mouse anti-FLAG (Sigma). After 48 h incubation at 4°C, the wells were blocked in blocking buffer (TBS, 0.05% [v/v] Tween 20, 5% [w/v] skim milk powder) for 2 h at 37°C. Sera, diluted in TBS/5% skim milk, were added to the wells and incubated for 2 h at 37°C. Wells were then washed 4× with blocking buffer and incubated for 2 h at 37°C with an anti-rabbit Ig horseradish peroxidase conjugate (Silenus) followed by ABTS (2,2-azino-*bis* 3 ethyl-benzothiazoline-6-sulfonic acid; Sigma), 1 μg/ml plus hydrogen peroxide (0.03% v/v) in acetate buffer (sodium acetate 100 mM, NaH_2PO_4 50 mM) pH 4. Color development was read at 405 nm after 20 min incubation at room temperature. Titers are expressed as the reciprocal of the endpoint dilution. This was defined as the final dilution giving an optical density of at least 0.1 units above the optical density obtained from 1:50 dilution of the preimmune serum. This was less than 0.1.

Histology

Ovaries from rabbits immunized with Ur-rZP2 or Ur-rZP3 were fixed in either 2% (w/v) paraformaldehyde or Bouin's solution, paraffin-embedded, sectioned at 4 μm, and then stained with hematoxylin and eosin.

Immunostaining of Frozen Ovary Sections

Ovaries to be frozen were removed from rabbits at autopsy and submerged in optimal cutting temperature (OCT) embedding medium in a cryomould on dry ice. Sections of frozen ovary tissue were cut at 5 μm using a cryostat and placed onto poly-lysine-coated glass slides. The tissue sections were air dried overnight before fixing with reagent-grade acetone for 5 min, then air dried again. Sections were blocked with 3% (w/v) BSA in PBS (pH 7.2), then incubated with the primary antibody diluted in 1% (w/v) BSA/PBS for 2 h at room temperature. After washing the sections with PBS/0.05% v/v Tween 20, the sections were incubated with a secondary anti-rabbit IgG-FITC or anti-mouse FITC-conjugated antibody diluted in 1% w/v BSA in PBS. Immunofluorescence was detected using a Bio-Rad MRC 1000 confocal microscope or by standard fluorescent microscopy. For detection of serum antibodies to rZP3 from infected rabbits, ovarian sections from uninfected animals were incubated with preimmune or immune sera diluted 1/100 followed by anti-rabbit Ig-FITC-conjugated antibody diluted 1/300 (Chemicon). Anti-rZP3 antibodies bound to zona pellucida in ovaries from infected animals were detected by incubating the sections with anti-rabbit Ig-FITC (1/300). Ovarian sections from uninfected rabbits were used as negative controls. For detection of macrophages or T lymphocytes, ovarian sections were incubated with mouse monoclonal antibodies L12/201 or L11/35 (Serotec), which detect rabbit CD45 and CD43 respectively, diluted 1/200 followed by anti-mouse FITC-conjugated antibody at 1/100 dilution.

Rabbit Inoculations and Infections

All animal experimental procedures were approved by the CSIRO, Division of Sustainable Ecosystems, Gungahlin, Animal Experimentation Ethics Committee in accordance with National Health and Medical Research Council/CSIRO guidelines.

Laboratory rabbits (*Oryctolagus cuniculus*), 6–12 mo old, bred at the Gungahlin animal facility, were housed in individual cages in a temperature-controlled room under physical containment level 2 conditions approved by the Commonwealth of Australia Office of the Gene Technology Regulator. Recombinant myxoma viruses Ur-rZP2, Ur-rZP3, Ur-rZP3-gpt/gus or Ur-rZP3-anc-gpt/gus were diluted in PBS (pH 7.4), and 1000 pfu inoculated intradermally over the thigh in a volume of 100 μl. Animals were killed before autopsy by intravenous injection of 1 ml/2 kg body weight of 325 mg/ml pentobarbitone.

Fertility Trials

Infected females were mated with proven fertile male rabbits at 30 or 35 days after infection, by which time all rabbits had recovered from the clinical disease induced by infection. Each female was placed with a proven-fertile male until mating was observed, and the female was then paired with a second male and left overnight. If mating was not observed with the first male, the female was left with this first male overnight and then placed with a second male overnight.

Fertility Trials with Ur-rZP2 and Ur-rZP3

Ur-rZP2 was tested in six female domestic rabbits; the animals were killed 10 days after mating and developing fetuses were counted to determine potential litter sizes. Ur-ZP3 was initially tested in 11 rabbits; fetuses were counted at autopsy 10 days after mating. A longer-term fertility trial was subsequently performed with Ur-ZP3 whereby 12 rabbits were mated and fertility was ascertained by the birth of offspring. Nesting boxes and nesting material were provided 4 wk postmating, and boxes were checked twice daily for nest-building and litters. The rabbits were mated again 1 wk after the last litters were born, followed by a third mating after the second round of breeding. Ten days after the third mating, all rabbits were autopsied and potential litter sizes were determined by counting the developing fetuses.

Fertility Trials to Test Dose Responsiveness to Ur-rZP3

Two groups of six female laboratory rabbits were infected with 100 or 10 000 pfu of Ur-rZP3, mated at 30 days after infection and autopsied 10 days after mating.

Fertility Trials with Ur-rZP3-gpt/gus and Ir-rZP3-anc-gpt/gus

Ur-rZP3-gpt/gus was tested in 12 female rabbits that were mated at 35 days after infection and then autopsied 10 days after mating. Ur-rZP3 anc-gpt/gus

was tested in six female rabbits that were mated 35 days after infection and autopsied 10 days later.

Statistical Analysis

To minimize the unnecessary use of animals, three approaches were used to determine control data. First, normal mating data for this laboratory rabbit breeding colony were analyzed. Over an 8-yr period involving 418 matings of 84 females, the litter rate per mating was 91% and the average litter size was 6. Using this littering rate as a basis for pregnancy rates, the probability of different numbers of pregnancy in groups of 6, 11, and 12 test rabbits (the group size we have used) were calculated using the binomial distribution (Table 1). A significant reduction in pregnancy rate for 12 rabbits would occur if 8 or fewer were pregnant ($P < 0.05$) or 6 or fewer ($P < 0.001$). For 11 rabbits, the numbers for significance are 7 and 5, and for 6 rabbits, 3 and 1. Second, each experiment was compared with the other experiments so that Ur-rZP2, Ur-rZP3, Ur-rZP3-gpt/gus and Ur-rZP3-anc-gpt/gus were each compared to each other. Third, an uninfected control group of six female rabbits was mated and autopsied 10 days after mating to determine pregnancy.

Cellular Immune Response Time Course Trial

Female rabbits were injected with 1000 pfu of Ur-rZP3, and then at Days 0, 5, 10, 15, 20, 35, and 60 after infection, three rabbits were killed. The ovaries were removed for histological examination; the right ovary was fixed in Bouins' solution and the left ovary frozen in OCT compound on dry ice. The spleen was removed from each animal to prepare primary lymphocyte cultures. Lymphocytes were cultured in RPMI with10% (v/v) fetal calf serum at 37°C in an atmosphere of 5% CO_2/95% air.

T-Cell Proliferation Assay

Primary cultures of rabbit spleen cells were dispensed at a density of 3×10^5 cells per well of five replicate 96-well plates. FLAG-rZP3 was added at four different dilutions (1/10, 1/25, 1/100, and 1/200) in PBS (pH 7.2) of a 100 μg/ml stock. An equivalent volume of PBS replaced the antigen in negative controls. Concanavalin A (ConA) was added to positive control wells at a final concentration of 5 mg/ml. Each experimental treatment was performed in quadruplicate wells. ^{3}H-thymidine (1 μ Ci/well in 50 μl RPMI) was added to the plates at Days 2, 3, 4, 5, and 6 after antigen addition. After ^{3}H-thymidine addition, each plate was incubated for 24 h before freezing. Cells were harvested on a Packard Cell harvester and ^{3}H-thymidine incorporation determined using scintillation counting. The stimulation index was calculated by dividing the average counts per minute (CPM) of the wells where antigen was added by the average CPM of wells where no antigen was added.

Testing for DTH Responses

Six female rabbits and six male rabbits were infected with 1000 pfu of Ur-rZP3. At 15 days after infection, 100 μg of FLAG- rZP3 antigen in a volume of 50 μl was intradermally injected into a clearly demarcated, shaved region over the thigh for three female and three male rabbits. A control inoculation was administered using the same buffer as the antigen inoculation, within a shaved area of the thigh easily distinguishable from the rZPC inoculation site. The inoculation sites were inspected at 24 and 48 h postinoculation and signs of redness and/or inflammation recorded. At 30 days after infection, the remaining three male and three female rabbits were tested and the original rabbits retested for DTH response by the inoculation of FLAG-rZP3.

TABLE 1. Probabilities of pregnancy outcomes for 12, 11, and 6 test rabbits based on the breeding colony pregnancy rate of 91%.

	Probability that n or fewer rabbits out of N are pregnant		
n	N = 12	N = 11	N = 6
12	1.0000		
11	0.6780	1.0000	
10	0.2950	0.6460	
9	0.0870	0.2600	
8	0.0180	0.0700	
7	0.0027	0.0130	
6	0.0003	0.0017	1.0000
5	0	0.0002	0.4320
4	0	0	0.0950
3	0	0	0.0120
2	0	0	0.0008
1	0	0	0
0	0	0	0

RESULTS

In Vitro Expression of rZP2 and rZP3 by Recombinant Myxoma Viruses

Recombinant myxoma viruses expressing rZP2 and rZP3 respectively were shown to be free from wild-type virus by PCR (data not shown). Both Ur-rZP2 and Ur-rZP3 were shown to express the respective protein by immunoblotting (data not shown).

Clinical Signs During Infections with Ur-rZP2 or Ur-rZP3

Rabbits infected with Ur-rZP2 developed a large primary lesion at the inoculation site but only mild clinical signs of myxomatosis. Thus, this virus was highly attenuated compared to the parental Ur strain [39]. Mild to moderate clinical signs of myxomatosis were observed in rabbits infected with Ur-rZP3. These were characterized by a primary lesion at the site of infection, secondary lesions around the eyes, ears, and nose, and mild anogenital redness and swelling. Over a test period of 30 days, infected rabbits had an elevated temperature $\geq$ 40°C for 23 rabbit days (calculated by the number of animals with rectal temperature $\geq$ 40°C $\times$ the number of days at this temperature). This is much lower than observed for the nonrecombinant parental strain, in which, using the same size test group, there were 139 rabbit days at a temperature $\geq$ 40°C [10]. This indicated that Ur-rZP3 was also attenuated in virulence in comparison to the parental Ur strain of virus.

The Effects of Infection with Ur-rZP2 on Fertility and Antibody Responses to rZP2

All six rabbits infected with Ur-rZP2 were pregnant, with 2 to 10 implantations per rabbit (mean 6.3, SD 2.9). This pregnancy rate was not significantly different from the predicted pregnancy rate of 91% (Table 1) and potential litter size was not different from the average for the breeding colony (six). Figure 2A shows that for all six rabbits, anti-rZP2 antibodies were present from 14 days after infection with titers of 12 800 and higher at days 28–35. No anti-rZP2 antibodies were detected in serum before infection with the recombinant virus. These serum antibodies bound to rabbit zona pellucida in ovarian sections (Fig. 2B).

The ovaries from rabbits infected with Ur-rZP2 were histologically normal with no obvious differences to age-matched controls (data not shown). Because Ur-rZP2 did not induce any infertility in this pilot trial, no further fertility trials were performed with this antigen.

The Effects of Infection with Ur-rZP3 on Fertility

Eleven female rabbits were infected with Ur-rZP3, mated 30 days after infection, and killed 10 days after mating. Three of the 11 rabbits were pregnant, with one, three, and four implantations. Eight were not pregnant. This was significantly less than the predicted success for a group of 11 rabbits ($P < 0.001$; Table 1). Nonpregnant animals had normal corpora lutea, indicating that ovulation and mating had occurred.

To determine the duration of infertility, 12 female rabbits were infected with Ur-rZP3 and mated 30 days later. These rabbits were allowed to give birth; seven rabbits had no

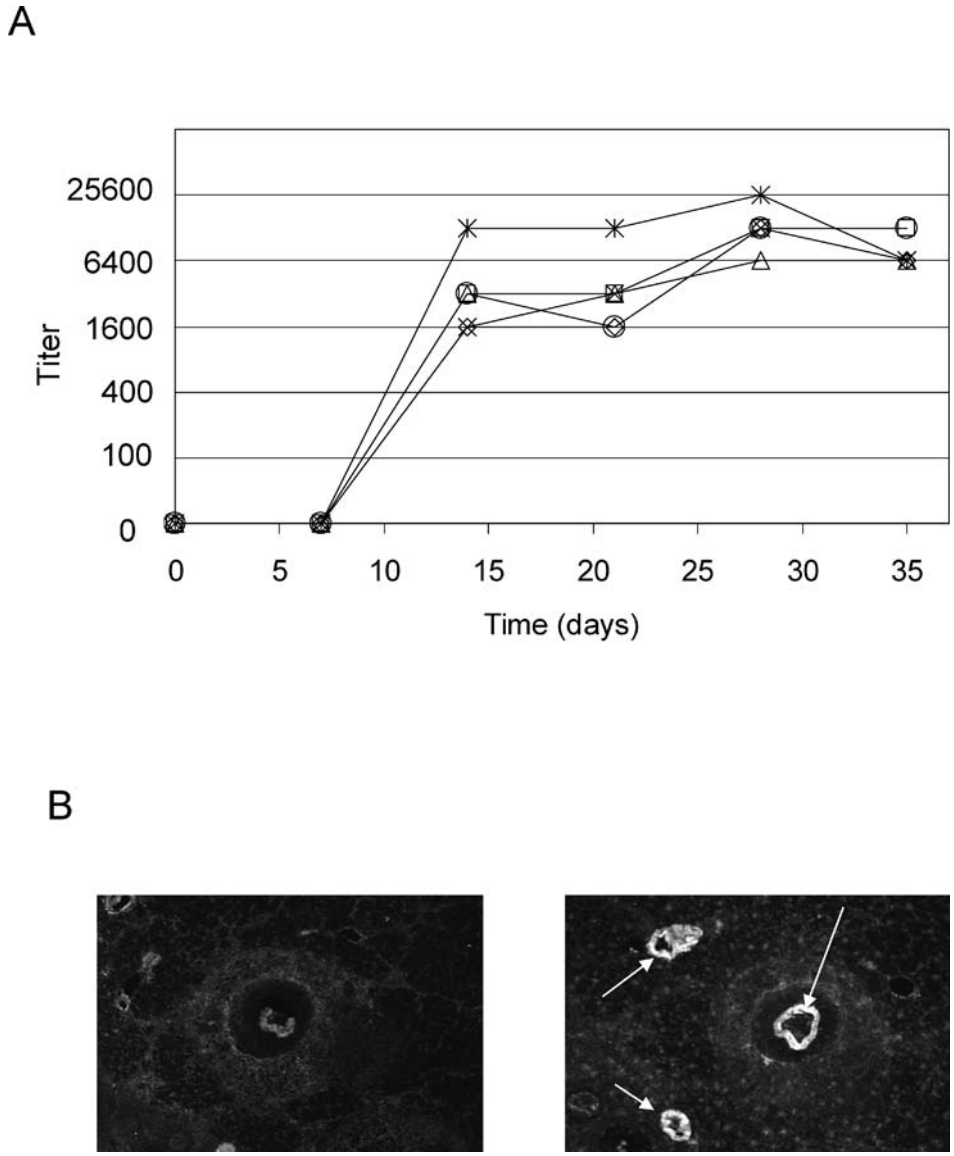

FIG. 2. Antibody responses against rZP2. **A**) Serum antibody titers to rZP2 measured by ELISA for six individual rabbits in a short-term fertility trial. **B**) Serum antibody binding to zona pellucida in ovarian follicles. Left-hand side shows fluorescent microscopy image of normal rabbit ovarian section probed with preimmune serum at 1/100 dilution followed by anti-rabbit Ig-FITC. Right-hand figure shows a section probed with Day 14 serum from a rabbit immunized with Ur-rZP2 diluted 1/100 followed by anti-rabbit Ig-FITC. Arrows indicate antibody bound to zona pellucida. Original magnification ×100.

offspring, whereas five had litters ranging from two to eight kittens (mean 4, SD 2.3). This pregnancy rate is significantly lower than that predicted for the colony for a group of 12 rabbits ($P < 0.001$; Table 1). However, following a second mating, only one rabbit remained infertile, and 11 had litters ranging from two to ten kittens (mean 7.1, SD 2.6). This pregnancy rate is not different from the predicted rate for the colony (Table 1). At autopsy after the third breeding, only the previously infertile animal was not pregnant. The remaining 11 rabbits were pregnant, with potential litter sizes of between six and thirteen (mean 9.4, SD 2.0). This pregnancy rate was not different from the colony (Table 1).

Serum Antibody Responses to rZP3

Serum antibody titers to rZP3 were highest at Day 15 after infection between 800 and 6400. Rabbits that were fertile at the first breeding had peak titers of 800 to 3200, whereas infertile rabbits had peak titers from 800 to 6400. Seven of the 12 animals still had detectable antibody titers between 100 and 800 at the final time-point (Day 86). Of the two rabbits with titers of 800 at Day 86, one (rabbit 009) remained infertile for three breeding cycles, whereas the other (rabbit 989) was infertile at the first breeding, had two kittens following the second breeding, and had nine implantations after the third breeding. Figure 3B shows the geometric mean titers for all rabbits and for the rabbits that were fertile or infertile at the first breeding. Comparison at Days 15, 20, and 27 revealed no significant differences between the titers of fertile and infertile rabbits ($P > 0.05$, two-tailed Mann-Whitney U-test). There was a strong linear decay of mean antibody titer against time, with a half-life of around 20 days. Comparison of Figure 3B with Figure 3A shows that individual rabbits have somewhat different decay curves. Serum antibodies to rZP3 from these animals bound to zona pellucida in normal ovarian sections (Fig. 3C).

Ur-rZP3 was also used to infect three male rabbits. Antibody was not detectable at Day 8, but between Days 14 and 28 the rabbits had titers of 800, 1600, and 1600. For two of the rabbits, these titers were stable at 800 until Day 56 when the final sample was taken. For the other rabbit, the titer had dropped to below detectable limits (<50) by this time. Thus, even though rZP3 is a self-antigen in female rabbits and a foreign antigen in male rabbits, there was no difference in antibody responses to rZP3 in males compared to females.

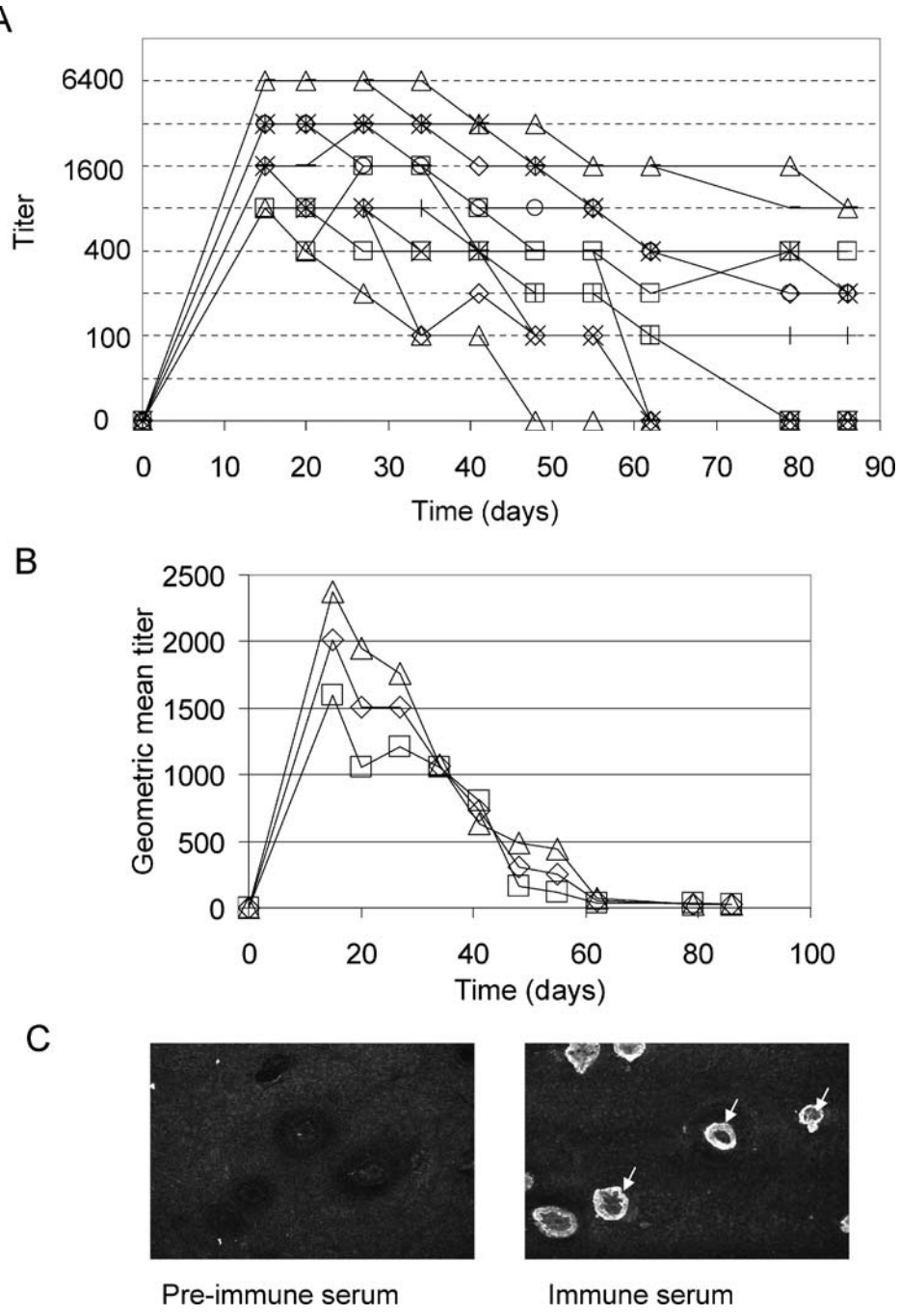

FIG. 3. Antibody responses to rZP3. **A**) Serum antibody titers to rZP3 measured by ELISA for 12 individual females in a long-term fertility trial. **B**) Geometric mean serum antibody titers for the 12 rabbits shown in **A**. Diamond indicates geometric mean titers for all 12 rabbits; square indicates geometric mean titers for fertile rabbits; triangle indicates geometric mean titers for infertile rabbits. **C**) Serum antibody binding to zona pellucida in ovarian follicles. Left-hand side shows fluorescent microscopy image of normal rabbit ovarian section probed with preimmune serum at 1/100 dilution followed by anti-rabbit Ig-FITC. Right hand figure shows a section probed with Day 15 serum from a rabbit immunized with Ur-rZP3 diluted 1/100 followed by anti-rabbit Ig-FITC. Arrows indicate antibody bound to zona pellucida. Original magnification ×100.

Effect of Viral Dose of Ur-rZP3 on Fertility

Two groups of six female rabbits were immunized with 100 pfu or 10 000 pfu of Ur-rZP3, respectively. These rabbits were mated 30 days after infection, and pregnancy rates were assessed at autopsy 10 days after mating. Four of six rabbits inoculated with 100 pfu of Ur-rZP3 and four of six rabbits inoculated with 10 000 pfu were infertile, which was significantly less than the predicted fertility rate ($P < 0.001$; Table 1). Potential litter size of the fertile rabbits was similar for each viral dose. A third group of six females was mated as uninfected controls, and five of six were pregnant. This was not different from the predicted pregnancy rate for uninfected animals (Table 1). Thus, there was no dose-response to the virus in terms of proportion of rabbits that failed to litter across a dose range of two orders of magnitude.

Testing the Immune Response to rZP3 Not Presented on the Cell Membrane of Infected Cells

Monolayers of RK13 cells were infected with Ur-rZP3-anc-gpt/gus, which has the membrane anchor sequence of rZP3 deleted, or the control virus Ur-rZP3-gpt/gus. Both viruses expressed rZP3 within the infected cells, but rZP3 was not detected on the surface membrane of cells infected with Ur-rZP3-anc-gpt/gus (Fig. 4).

Six of six rabbits infected with Ur-rZP3-anc-gpt/gus were pregnant (mean number of implantations 8.3; SD 2.1). However, only three of the 12 females infected with the control virus Ur-rZP3-gpt/gus were fertile (mean litter size 4.7; SD 4.0), which was significantly less than the predicted fertility rate ($P < 0.001$; Table 1) and similar to results obtained with the Ur-rZP3 recombinant virus. All infertile rabbits showed

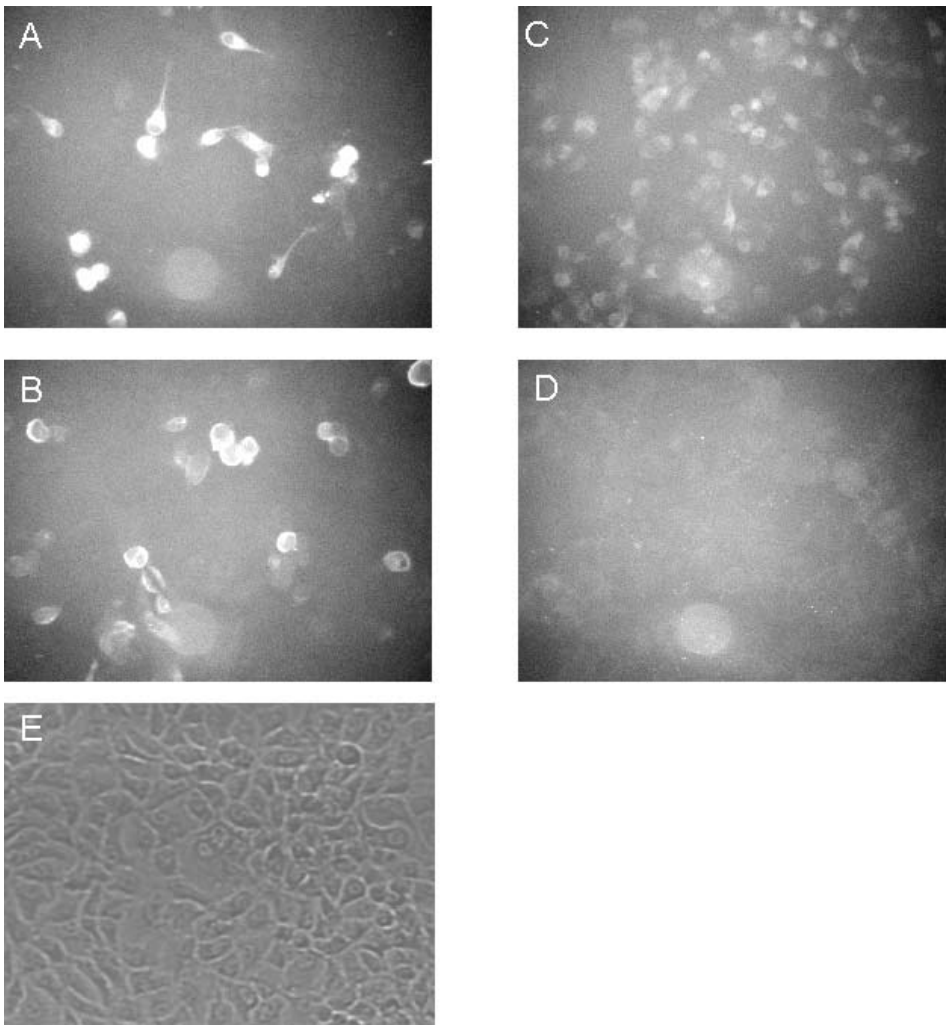

FIG. 4. Localization of rZP3 on the cell membrane. **A**) Fluorescent microscopy image of RK13 cells infected with Ur-rZP3-gpt/gus moi 1 at 16 hours after infection. Cells were fixed with methanol/acetone (1:1 v/v) to allow antibody entry and probed with a 1/400 dilution of rabbit anti-pig zona pellucida serum followed by anti-rabbit Ig-FITC. **B**) As for A, but fixed with 4% formaldehyde to demonstrate cell membrane bound rZP3. **C**) Fluorescent microscopy image of RK13 cells infected with Ur-rZP3-anc-gpt/gus moi 1 at 16 hours after infection. Cells were fixed with methanol/acetone (1:1 v/v) to allow antibody entry and probed with a 1/400 dilution of rabbit anti-pig zona pellucida serum followed by anti-rabbit Ig-FITC. **D**) As for C, but fixed with 4% formaldehyde to demonstrate lack of cell membrane bound rZP3. **E**) RK13 cells infected with Ur moi 1 at 16 hours after infection. Cells fixed with methanol/acetone (1:1 v/v) and probed with a 1/400 dilution of rabbit-anti-pig zona pellucida serum followed by anti-rabbit Ig-FITC. Original magnification ×200.

normal corpora lutea, indicating that mating had occurred and had induced ovulation.

Sera from the rabbits infected with Ur-rZP3-anc-gpt/gus were tested for binding to rabbit zona pellucida by immunofluorescent staining of normal rabbit ovaries. No binding of serum from these rabbits with zonae pellucidae was detected. Ovaries stained using serum from Ur-rZP3-infected females were strongly fluorescent (data not shown). However, when ovaries from the infected rabbits were stained with anti-rabbit Ig, follicles from two of six rabbits stained weakly, suggesting that a small amount of antibody to rZP3 may have been elicited by the infection. From this experiment, it appears that the context of antigen expression and presentation is critical to development of an autoimmune response to rZP3.

T-Cell Immune Responses to rZP3

Splenocytes were prepared from female rabbits infected with Ur-rZP3 at 0, 5, 10, 15, 20, 35, and 60 days after infection and stimulated in vitro using rZP3-FLAG. Lymphocytes proliferated normally in response to the T-cell mitogen ConA. However, antigen stimulation with rZP3-FLAG did not induce any cell proliferative response at any time point (data not shown).

A separate group of rabbits was tested for delayed-type hypersensitivity T-cell responses by skin inoculation of rZP3-FLAG at 15 and 30 days after immunization with Ur-rZP3. Neither male nor female rabbits showed significant DTH responses at the skin challenge sites (data not shown).

The Effects of Infection with Ur-rZP3 on Ovarian Histology

Ovarian pathology was examined from three experiments: (1) Short-term mating trials, in which the ovaries were collected 10 days after mating, i.e., Day 40 after infection with Ur-rZP3. (2) Long-term mating trials, in which ovaries where collected 10 days after the third mating, i.e., approximately 100 days after infection. (3) Time-course trials, in which ovaries were collected at Days 0, 5, 10, 15, 20, 35, and 60 after infection but no matings were done.

Ovaries collected 10 days after mating, i.e., 40 days after infection, from rabbits infected with Ur-rZPC appeared abnormal. All sections contained populations of primordial, primary, and preantral secondary follicles (47–272 follicles/section) consistent with age-matched uninfected controls (89–140 follicles/section). However, some of these preantral secondary follicles showed abnormal formation characterized by disorganized granulosa cells, the presence of granulosa cells within the zona pellucida, and abnormal zona formation (Fig. 5, A, C, and E) when compared to controls (Fig. 5, B, D, and F). The majority of the ovarian sections contained large numbers of antral follicles (0–20/section) but these follicles had a noticeably increased rate of apoptosis of the granulosa cells (>95%) plus aberrant formation of the zona pellucida and zona pellucida with missing oocytes (Fig. 5, G and I). Only 22% of

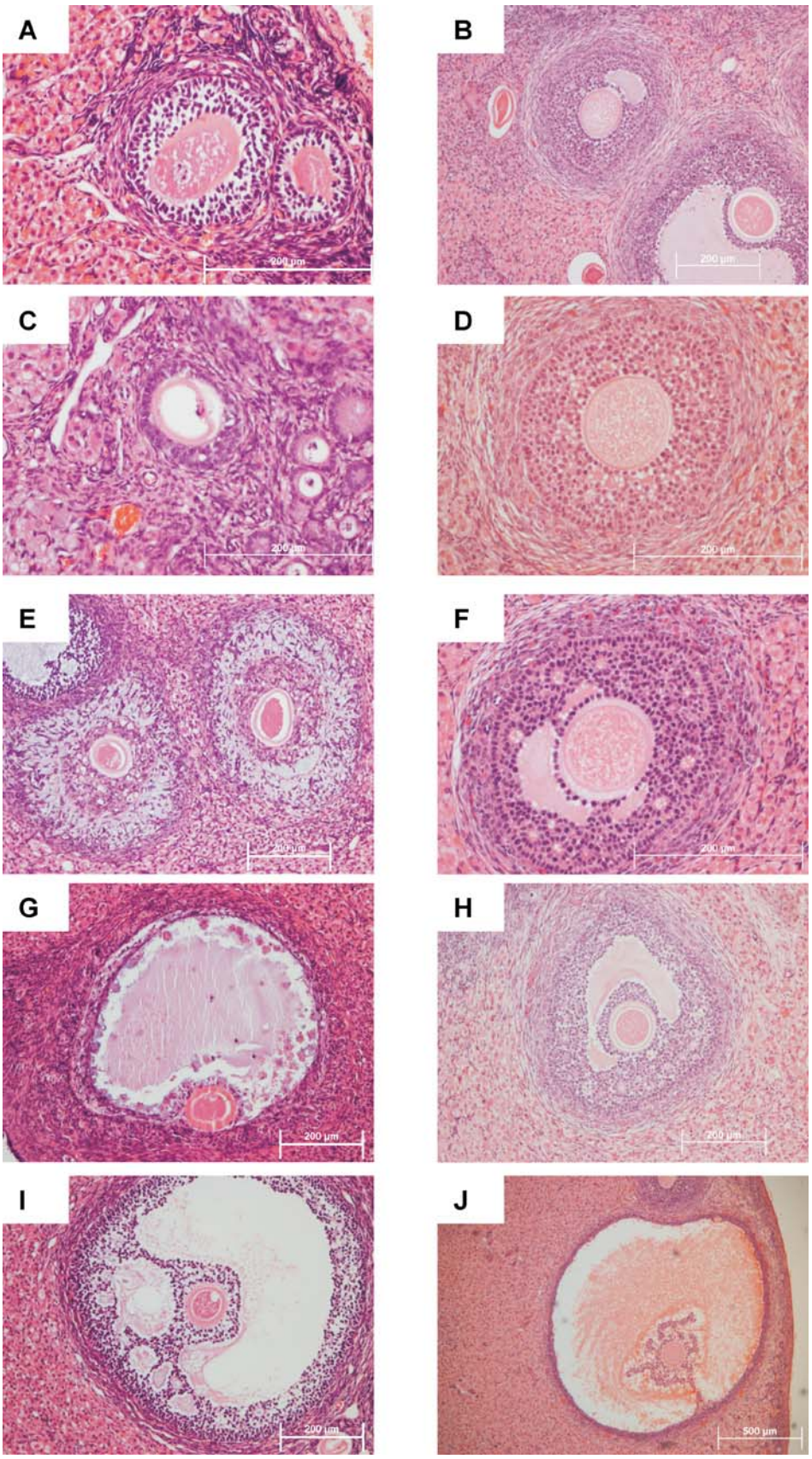

FIG. 5. Morphology of ovaries from rabbits infected with Ur-rZP3. **A**) Aberrant zona pellucida and presence of granulosa cells within the zona pellucida of an immunized rabbit. **B**) Normal secondary follicles in an age-matched control (mated) animal. **C**) Zona pellucida with missing oocyte in immunized animal. **D**) Normal secondary follicle in age-matched control (mated) animal. **E**) Aberrant granulosa cell proliferation in follicles from immunized rabbit. **F**) Normal secondary antral follicle from fertile rabbit. **G** and **I**) Apoptotic granulosa cells and oocyte degradation in follicles from immunized rabbits. **H** and **J**) Normal antral follicles from fertile control rabbit. Bars = 200 µm (**A–I**) and 500 µm (**J**).

large antral follicles were apoptotic in the age-matched controls (Fig. 5, H and J). There were no obvious histological differences between pregnant and nonpregnant rabbits.

Histologically, the majority of ovaries collected at the end of the long-term fertility trial appeared normal, with healthy antral follicles and ovulation indicated by the presence of corpora lutea and multiple corpora albicans. Approximately 30% of the large follicles appeared apoptotic, which is to be expected in newly pregnant animals and was similar to control sections. Similarly, remnants of follicles indicated previous waves of follicular development. Most sections contained normal numbers of healthy primordial, primary, and early secondary follicles. However, the ovaries from two animals were distinctly different from the others. Rabbit 009, which never became pregnant, had no evidence of active corpora lutea, but, based on the presence of a corpus albicans in one ovary, it had ovulated during the trial. There were no antral follicles but normal numbers of primordial follicles. Primary and secondary follicles were few in number (<27/section) and had aberrant morphology.

Ovarian pathology was further examined over a 60-day time course. Rabbits were infected with 1000 pfu of Ur-rZP3 and ovaries harvested from each of three rabbits at Days 0, 5, 10, 15, 20, 35, and 60. These rabbits were not mated at any time. Histological changes were present only at Day 15 and were limited to a loss of large viable antral follicles with normal numbers of primordial, primary, and secondary follicles. Sections from Day 10 or Day 20 rabbits had normal numbers of antral follicles (range 1–6 per section), as did rabbits from all other timepoints.

Cells staining positively for CD45, by immunofluorescence on frozen sections, clustered around some of the larger secondary follicles at Day 15. The distribution of these cells ranged from completely surrounding the follicle (Fig. 6) to, more commonly, just a few positive cells at the periphery of the follicle. This staining was quite intense in two of the three rabbits at this time point, whereas the third rabbit had fewer positive cells scattered around the large secondary follicles and the staining was not as intense. Positive cells were also scattered throughout the rest of the ovary at Day 15. At Day 20, one of three rabbits had staining around a few of the large secondary follicles, but this was limited to one or two positive cells per follicle. At other time points, including the Day 0 controls, CD45 cells were generally uncommon but, if present, were scattered throughout the ovary and were not clustered around the follicles (data not shown). No sections stained positively for CD43, indicating that the CD45-staining cells were of the macrophage lineage rather than T lymphocytes (data not shown).

Antibody bound to zonae was detected as early as Day 10 in one of three rabbits; at Day 15 all three rabbits showed positive staining. Antibody bound to the zonae was very prominent by Day 35 after infection and persisted for the 60 days of the experiment (Fig. 7).

DISCUSSION

The recombinant virus Ur-rZP2 had no effect on fertility. This was despite all infected rabbits producing serum antibodies against rZP2 protein, which bound to the zona pellucida in ovarian sections. Conversely, 24 of 35 rabbits (69%) infected with Ur-rZP3, at three different virus doses, were infertile for at least the first breeding. Similar levels of infertility were obtained with the independently constructed Ur-rZP3-gpt/gus recombinant virus, with 9 of 12 female rabbits (75%) infertile following a single mating. Together, 33 of 47 rabbits (70%) were infertile. An overall fertility rate of 30% following immunization with Ur-rZP3 is significantly less than expected ($P < 0.001$). In a group of six uninfected control rabbits, 5 of 6 became pregnant, which is not different from the colony breeding success rate. However, in the long-term fertility trial with Ur-rZP3, at each of the second and third matings 11 of 12 rabbits (92%) were pregnant, which is consistent with the long-term breeding success in the colony. All of these matings used the same male rabbits. Thus, immunization with Ur-rZP3 induced a significant reduction in fertility in female laboratory rabbits, but this was transient, with return to normal fertility within 2 mo.

Infertility was not caused by virus infection, because when rabbits were infected with either Ur-rZP2 or Ur-rZP3-anc-gpt/gus, 6 of 6 rabbits were fertile. This confirmed the overall high success rate for mating in the colony and also indicated that infection with a recombinant virus alone was not sufficient to induce transient infertility. Similarly, previous breeding trials in this colony with females immunized with Ur-rZP1 have shown no difference to the colony breeding success rate following mating at 35 days after immunization [10]. In the same trial, when control siblings were infected with the parental Ur strain of myxoma virus, 8 of 8 rabbits were pregnant following the first mating. These results strongly support a hypothesis that infection with attenuated myxoma virus alone does not induce infertility in female rabbits. It is not possible to exclude a role for a myxoma virus-induced systemic proinflammatory state in ovarian pathology, even though myxoma virus does not actually replicate in rabbit ovaries [11].

Infertility following infection with Ur-rZP3 could be attributable to failure of follicular maturation and oocyte viability, endocrine dysfunction, interruption of egg-sperm binding or sperm penetration and fertilization, or failure of embryo transfer and implantation (reviewed in [16]). In the current study, there was only limited evidence of ovarian pathology or inflammation, and this was not different between fertile and infertile rabbits. The rabbits had ovulated on mating, as indicated by the development of apparently normal corpora lutea. This suggests that endocrine function was retained. Follicular loss was not different between fertile and infertile animals, showing that there was a very fine balance between

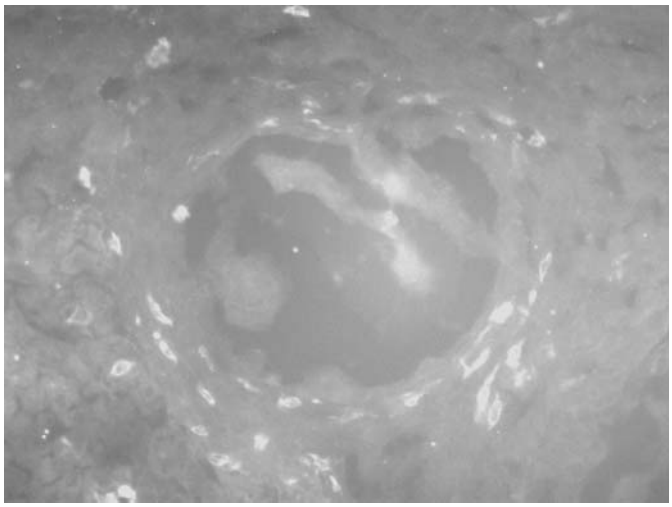

FIG. 6. Immunofluorescent staining for CD45 expression in rabbit ovary. Frozen ovarian sections from rabbits infected with Ur-rZP3 were probed with a monoclonal antibody to rabbit CD45 followed by anti-mouse IgG-FITC. Section shows a follicle surrounded by CD45 positive cells at 15 days after infection. Original magnification ×400.

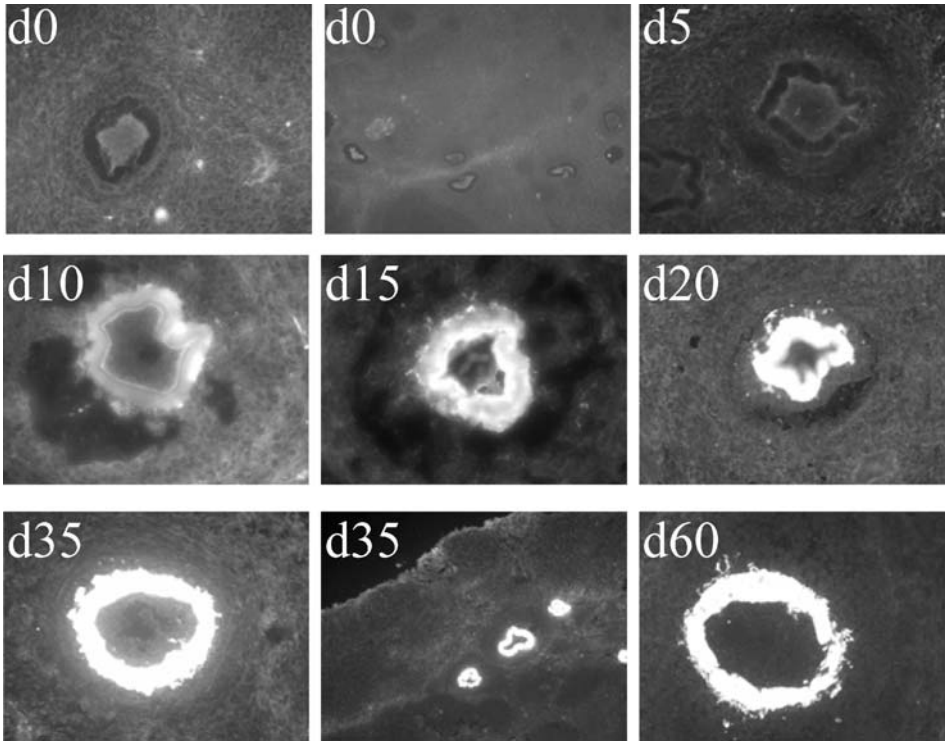

FIG. 7. Immunofluorescent detection of rabbit immunoglobulin bound to zona pellucida in ovaries from rabbits infected with Ur-rZP3. Ovaries were collected at Days 0, 5, 10, 15, 20, 35, and 60 from four rabbits at each timepoint and frozen sections probed with anti-rabbit Ig-FITC to detect immunoglobulin bound to the zonae. Typical high power sections are shown for each timepoint (original magnification ×400). For Days 0 and 35, both high-power and low-power (original magnification ×100) sections are shown.

fertility and infertility and indicating that it is not possible to superficially conclude that follicular failure or production of nonviable oocytes was responsible for the infertility that occurred. Induction of serum antibodies that cross-react with zona pellucida is not sufficient to induce infertility, because only rZP3 has consistently induced significant infertility when delivered by a recombinant virus, even though ZP2 and ZP1 induced antibodies that bound to the zona pellucida. Infection with recombinant myxoma virus expressing rZP3 induced follicle loss or failure of follicle maturation associated with a transient inflammatory response around the follicle and serum antibodies to zona pellucida that are bound to the zona pellucida in secondary and larger follicles. However, it is not proven that any of these features are the key to the mechanism of infertility. The antibody response to rZP3 was relatively short-lived, as was the infertility and any ovarian pathology. It is possible that the CD45+ cellular response in the ovaries around Day 15 after immunization actually has a regulatory role in suppressing presentation of native ZP products from the follicles by dendritic cells either locally or at the draining lymph node and thus maintaining the immune-privileged nature of the ovary.

Myxoma virus encodes a series of molecules that suppress innate and cellular immune responses in rabbits [40]. Despite this immunosuppressive nature of myxoma virus, infection of rabbits induces high titered serum IgG against myxoma virus that persists for at least 2 yr and probably for the life of the rabbit [41]. Similarly, immunization of rabbits with a recombinant myxoma virus expressing influenza hemagglutinin induced high-titered, long-lived serum IgG antibodies to both HA and myxoma virus [8, 9]. This is consistent with a model for antibody responses mediated by an initial burst of B cell stimulation, germinal center formation, and B cell differentiation into short- and long-lived plasma cells with concomitant production of B memory cells requiring CD4+ T cell help. Serum antibody titers would be maintained by persistent long-lived plasma cells or by B memory cells either stimulated by retained antigen complexes on follicular dendritic cells or potentially being constantly stimulated by polyclonal or bystander activation to differentiate into plasma cells [42].

In contrast, the antibody responses to the self-antigen ZP3 following inoculation with Ur-rZP3 were short-lived in most animals. Five of 12 of the rabbits had undetectable antibody titers to rZP3 by 86 days after immunization. This rapid loss of antibody resembled the decay curve of passively transferred maternal antibodies in rabbit kittens where antibodies to myxoma virus had fallen below detectable titers by 65 days after birth [41]. Given that passive maternal antibody has a peak titer at birth and the peak titers to rZP3 were at Day 15 these decay rates are very similar. This suggests that if long-lived plasma cells or constantly stimulated B memory cells existed then they were at very low levels.

The failure to detect a T-cell response to rZP3 in the presence of an antibody response suggests that peripheral immune tolerance to zona pellucida antigens may be maintained at the T-cell level rather than at the B-cell level. It is possible that CD4+ T cells to viral proteins were able to provide help to B cells that recognized rZP3 as part of a complex of rZP3 and viral proteins. Uptake of this complex by B cells with subsequent presentation of viral peptides on MHC-II molecules to primed T cells may have provided sufficient stimulation for B-cell differentiation and development of plasma cells. An alternative is that antibodies to rZP3 are being generated by a T-cell-independent response with no generation of B memory cells. It is surprising that rZP3 did not generate a persistent immune response in males, in which it is a foreign antigen, and that the kinetics of the antibody response were not different from those in female rabbits where rZP3 is a self-antigen. However, the antibody response was also similar in male and female rabbits immunized with Ur-rZP1 virus [10].

The data presented in this paper indicate that rZP3 has the potential to be developed as an immunocontraceptive antigen for rabbits. Further studies will need to focus on developing long-lived serum antibody responses to rZP3 and appropriate T-cell stimulation. Rabbit ZP3 provides the best model so far

for studying autoimmune infertility delivered by a recombinant myxoma virus.

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