BIOLOGY OF REPRODUCTION 58, 152–159 (1998)

Infertility in Mice Induced by a Recombinant Ectromelia Virus Expressing Mouse Zona Pellucida Glycoprotein 3[1]

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ABSTRACT

Population control has become a major problem in many wildlife species. Fertility control through immunocontraception has been proposed as a method for reducing population size. We have tested the concept that immunocontraception can be achieved with a recombinant ectromelia virus expressing an ovary-specific antigen, the mouse zona pellucida 3 (ZP3) glycoprotein. Female mice infected with the recombinant virus produced autoimmune antibodies against ZP3 and were infertile for 5–9 mo after infection. For almost half the infertile mice, immunity to ZP3 was associated with a disruption of ovarian follicular development and the depletion of mature follicles without observable oophoritis. Mice returned to fertility as the anti-ZP3 antibody level in the serum decreased. Reinfection of the mice with the recombinant virus boosted the anti-ZP3 response and restored infertility.

INTRODUCTION

Control of wildlife pest populations has usually relied on methods that increase mortality, such as disease, introduced predators, trapping, shooting, or poisoning. Many of these strategies are ineffective in the long term, and there is now growing public opinion that more humane methods should be examined. Fertility control through immunocontraception has been proposed as a method for reducing population size, especially for those species with high fecundity. However, where there are large numbers of a species occupying remote or broad areas of the environment, delivery of the infertility agent remains a major obstacle. The use of recombinant micro-organisms as vectors to deliver reproductive antigens to wildlife populations has been suggested [1].

One target for immunocontraception is the zona pellucida (ZP), the extracellular glycoprotein matrix surrounding mammalian oocytes, ovulated eggs, and early embryos. In the mouse, the ZP is composed of three non-covalently linked glycoproteins ZP1 (130 kDa), ZP2 (120 kDa), and ZP3 (83 kDa) [2]. The ZP proteins are unique to the ovary and are expressed by the growing oocytes [3, 4] within medium (types 3a, 3b, and 4) and large (types 5a and 5b) follicles [5]. The *O*-linked oligosaccharide of ZP3 mediates primary sperm binding to the ZP at the time of fertilization [6, 7]. The ZP also functions to block postfertilization polyspermy and to protect the preimplantation embryo as it passes through the oviduct [8]. The mouse ZP3 glycoprotein has been intensively studied as a target antigen for immunocontraception due to its crucial role in fertilization. Monoclonal antibodies directed towards ZP3 have been used to inhibit fertilization in vitro, and in vivo by passive immunization [9, 10]. Synthetic peptides encoding a ZP3 B-cell epitope have been used to actively immunize female mice, resulting in a variable period of immuno-infertility of between zero and eight months [11, 12].

Ectromelia virus is a natural pathogen of laboratory mice and causes the disease mousepox in susceptible strains [13]. Ectromelia virus is a member of the family Poxviridae, genus *Orthopoxvirus*, and is therefore closely related to vaccinia virus. We considered ectromelia virus to be an appropriate vector for a mouse vaccine because it is better adapted for replication in mice than vaccinia virus [14–16]. Because of the close genetic relationship, the recombination systems developed for vaccinia virus can be used to construct recombinant ectromelia viruses [17]. We have constructed a recombinant ectromelia virus expressing the entire mouse ZP3 cDNA for evaluation as a live recombinant immunocontraceptive vaccine for the mouse.

MATERIALS AND METHODS

DNA Manipulations

The plasmid pZP3.4 [18] contains a hybrid DNA sequence consisting of a mouse genomic fragment encoding exon-1 of the ZP3 gene ligated to a partial ZP3 cDNA [19] (Genbank Accession Number M20026) to generate a complete ZP3 open reading frame (ORF). A copy of the mouse ZP3 ORF lacking the 5′ untranslated sequences was generated by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and pZP3.4 as the template. The sense primer (5′-GGGATCCATGGCGTCAAGCTATTTCCTC) contained a *Bam*HI site positioned immediately upstream of the ATG initiation codon of the ZP3 protein coding sequence (underlined; nucleotide positions 30–50 of the ZP3 cDNA) while the antisense primer (5′-GGGAGATCTTTGCGGAAGGGATACAAGGTA) contained sequences complementary to the 3′ end (underlined; complementary nucleotide positions 1291–1281). A complete copy of the ZP3 ORF (nucleotide positions 30 [ATG] to 1294 [TAA]) was generated by ligation of the *Bam*HI-*Kpn* I fragment of the PCR product (nucleotide positions 30–435) to the *Kpn* I-*Eco*RI fragment of pZP3.4 (nucleotide positions 436 to 1317). The joined fragments were inserted between the *Bam*HI and *Eco*RI sites of pGem7Z(f−) (Promega Corporation, Madison, WI), generating plasmid pZP3. The DNA sequence of the *Bam*HI-*Kpn* I PCR fragment was determined and is identical to the published ZP3 cDNA sequence [19]. The ZP3 cDNA was excised from pZP3 using *Bam*HI and *Xho* I and inserted between the *Bam*HI and *Sal* I sites of the vaccinia virus vector pMJ602 [20], generating pMJ602-ZP3. In pMJ602-

Accepted September 2, 1997.
Received May 13, 1997.
[1]This research was supported by the Australian Government's Cooperative Research Centres Program.
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ZP3, the mouse ZP3 cDNA is positioned downstream of a strong synthetic poxvirus late promoter.

The vaccinia virus P11 late promoter was removed from pUrTK11 [21] by digestion with *Xba* I and *Hin*dIII and replaced with a *Xba* I-*Hin*dIII fragment isolated from p-UrTK14L [22] encoding a synthetic poxvirus late promoter and multiple cloning site, generating pUrTK11a. The mouse ZP3 cDNA was isolated from the pZP3 as above and ligated between the *Bam*HI and *Sal* I sites of p-UrTK11a (pUrTK11a-ZP3).

Cells and Viruses

The following cells were used: LM(TK−) (laboratory mouse [*Mus domesticus*], connective tissue fibroblast, thymidine kinase-deficient, ATCC: CCL-1.3); BS-C-1 (African Green Monkey [*Cercopithecus aethiops*] kidney epithelial ATCC: CCL-26); and RK13 (European rabbit [*Oryctolagus cuniculus*], kidney epithelial, ATCC: CCL-37). These were maintained in Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum (FBS). Ectromelia virus Moscow strain (ATCC:VRL-1374) was grown on LM(TK−) and BS-C-1 cells, and myxoma virus (Poxviridae, genus *Leporipoxvirus*) Uriarra strain [23] was grown on RK13 cells in MEM supplemented with 0.5% FBS.

Ectromelia virus isolated from homogenized infected tissues was titrated by growth on BS-C-1 cell monolayers in 6-well culture dishes. Plaques produced by recombinant viruses were visualized by using an MEM overlay containing 0.7% (w:v) agar and 300 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), and the blue staining plaques were counted. Plaques produced by wild-type ectromelia virus Moscow strain were detected by fixing and staining the infected cell monolayers with 20% (v:v) ethanol containing 0.1% (w:v) crystal violet.

Construction of Recombinant Viruses

Recombinant ectromelia viruses were generated by transfection of virus-infected LM(TK−) cells (0.2 plaque-forming units [pfu] per cell) using the plasmids pMJ602 or pMJ602-ZP3 according to standard procedures [24]. Ectromelia viruses with a thymidine kinase negative (TK−) phenotype were selected by adding 50 μg/ml 5-bromodeoxyuridine to the culture medium. Plaques containing recombinant viruses were confirmed by assay for β-galactosidase expression using a media overlay containing 0.7% (w:v) agar and 300 μg/ml X-Gal. Recombinant viruses ECTV-602 and ECTV-ZP3 were plaque-purified three times. Indirect immunofluorescence of virus-infected tissue culture cells [25] was used to demonstrate expression of recombinant mouse ZP3 (rZP3) by ECTV-ZP3. Confluent LM(TK−) cells were infected at a multiplicity of infection of 5 pfu/cell with either ECTV-ZP3 or ECTV-602. Twenty-four hours postinfection (p.i.) the infected cells were fixed with ice-cold 2% (w:v) paraformaldehyde in PBS to preserve cell surface proteins. The fixed cells were incubated with rat monoclonal antibody IE10 anti-mouse ZP3 [10] and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rat immunoglobulin (mouse adsorbed; Silenus Laboratories, Hawthorn, Victoria, Australia).

Immunoblotting [26] was also used to confirm the expression of rZP3 by ECTV-ZP3. LM(TK−) cells were infected as above with either ECTV-ZP3 or ECTV-602. At 24 h p.i. the cells were removed from the culture dishes using a cell scraper washed with PBS and then lysed in SDS-PAGE loading buffer. Proteins were separated by discontinuous SDS-PAGE, transferred to polyvinyl difluoride (PVDF) membrane, and then used for immunoblotting analysis using IE10 anti-mouse ZP3 and horse radish peroxidase (HRP)-conjugated anti-rat IgG (Southern Biotechnology Associates Inc., Birmingham, AL).

A recombinant myxoma virus (MYXV-ZP3) expressing the rZP3 glycoprotein was constructed using the transfer plasmid pUrTK11a-ZP3 as described previously [21]. Expression of rZP3 by MYXV-ZP3 was confirmed as described above (data not shown).

Mice, Vaccination, and Fertility Trials

Animal studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Specific pathogen-free 6- to 8-wk-old BALB/c mice, supplied by the Australian National University Animal Services Division, were inoculated with either 10^3 or 10^6 pfu of ECTV-ZP3 or ECTV-602 into the right hind foot-pad. Fertility trials were commenced 2 or 3 wk p.i. by pairing each female with a male for 2 wk. Vaginal plugs were monitored to confirm mating. For the experiment in which only a single pregnancy was monitored, the mice were killed after delivery of their litters or 3 wk after the removal of the male for those mice that were not pregnant. The uterus was examined to determine the number of implantation scars.

Histology and Immunofluorescence

Ovaries were collected from killed mice, fixed in Bouin's solution, and embedded in paraffin. Sections (7 μm) were stained with hematoxylin and eosin. Antibody bound in situ to ovarian ZP was detected by direct immunofluorescence. Ovaries were collected at 6 wk p.i. and snap-frozen in hexane in a dry ice-ethanol bath and embedded in OCT compound (Miles Inc. Diagnostics Div., Elkhart, IN), and 10-μm sections were prepared using a cryostat. Sections were fixed in 90% ethanol for 15 min and stained with FITC-conjugated anti-mouse immunoglobulin. For indirect immunofluorescence studies, ovaries were collected from uninfected mice and prepared as above. Sections were fixed in methanol for 5 min at −20°C, incubated for 1 h with antisera from mice recovered from infection with either ECTV-ZP3 or ECTV-602, and then stained with FITC-conjugated anti-mouse immunoglobulin.

Preparation of Soluble rZP3 Antigen for Immunoassays

The 8 genera of poxviruses infecting vertebrates are separated on the basis of genus-specific neutralizing antibodies [27]. Members of the genus *Orthopoxvirus* (e.g., ectromelia virus, vaccinia virus) do not share major cross-reactive antigens with the other poxvirus genera, including *Leporipoxvirus* (e.g., myxoma virus). Therefore, we used a recombinant myxoma virus (MYXV-ZP3) expressing rZP3 as a source of antigen for immunological assays since mouse sera containing anti-ectromelia virus antibodies show little immunoreactivity towards the myxoma virus proteins.

RK13 cells were infected for 24 h at 32°C with either a wild-type myxoma virus (control antigen) or MYXV-ZP3 at a multiplicity of 2 pfu/cell. The infected cells were detached from the culture flasks using a cell scraper and washed in PBS. For use in immunoblotting analysis, the infected cells were lysed in 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, and 2% (w:v) SDS, and heated at 95°C for 5 min; and the viscosity of the solution was reduced by

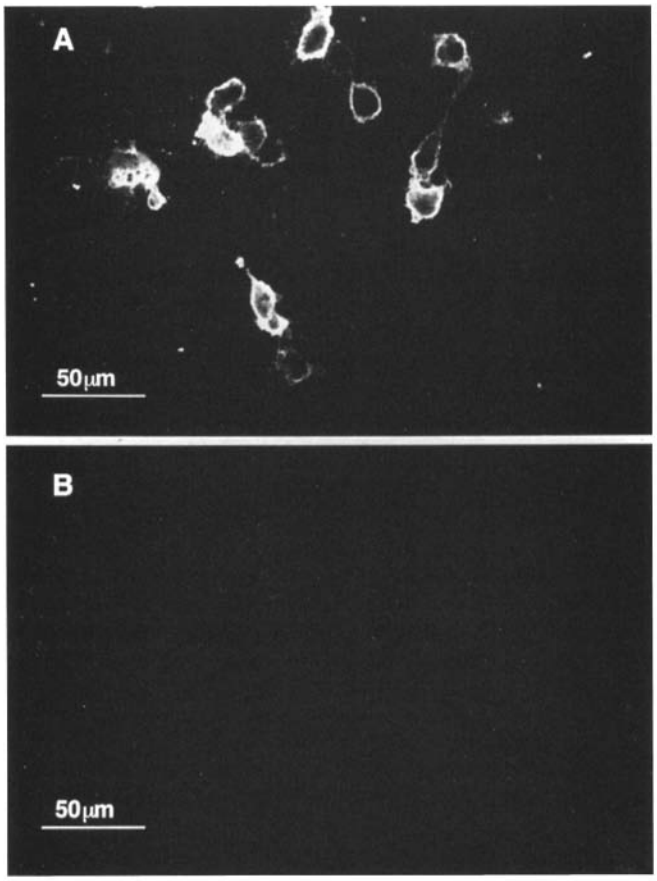

FIG. 1. Expression of rZP3 by ECTV-ZP3 infected tissue culture cells. Indirect immunofluorescence of LM(TK−) cells infected with recombinant virus ECTV-ZP3 (**A**) and ECTV-602 (**B**) were fixed to expose cell surface proteins and probed with monoclonal antibody IE10 rat anti-mouse ZP3.

repeated expulsion through a 22-gauge needle. For use in ELISA, the infected cells were lysed in 2% (v:v) Triton X-100, 1 μM leupeptin, 1 μM pepstatin, 0.1 μM aprotinin, 1 mM PMSF, and 1 mM EDTA in PBS; and the soluble protein extract was recovered.

Detection of Antibody to rZP3 by Immunoblotting

SDS-soluble proteins prepared from myxoma virus-infected RK13 cells were separated by discontinuous SDS-PAGE, transferred to PVDF membranes, and used for immunoblotting analysis of mouse sera (diluted 1:200) using standard procedures [26].

Detection of Antibody to rZP3 by ELISA

Each well of a 96-well round-bottom plate (Dynatech Laboratories Inc., Chantilly, VA) was coated with 200 ng/50 μl of Triton X-100-soluble rZP3 or control antigen (diluted in carbonate-bicarbonate buffer, pH 9.6) at 4°C overnight, then blocked with Blotto (PBS, 0.05% [v:v] Tween 20 [PBST] containing 5% [w:v] low-fat skim milk powder) for 1 h at room temperature. The mouse sera were preincubated in triplicate with control antigen (4 μg/ml in Blotto) for 1 h at room temperature to reduce nonspecific binding. The absorbed sera were diluted serially twofold in Blotto, added to the antigen-coated wells, and incubated overnight at 4°C. The assay plates were washed 5 times with PBST, incubated for 1 h with biotinylated sheep anti-mouse immunoglobulin (Amersham Corporation, Arlington Heights, IL), diluted 1:5000 in Blotto, and then incubated for 1 h with streptavidin-alkaline phosphatase conjugate (Amersham Corporation) diluted 1:2000 in Blotto, with washing as above after each step. The plates were incubated with *p*-nitrophenol phosphate substrate (Sigma Chemical Company, St. Louis, MO; 1 mg/ml in 0.01 M diethanolamine-HCl, pH 9.8) for 30 min, and the absorbance at 405 nm was determined with a microtiter plate reader. A standard curve was constructed with serial dilutions of a high-titer antiserum (defined arbitrarily at 1600 U/ml), which gave an endpoint dilution titer of 1:204 800 (using a cut-off value of mean background absorbance plus three times the standard deviation). Data were analyzed using the SOFTMAX computer program (Molecular Devices Corp., Menlo Park, CA). Background binding to control antigen-coated plates was < 10 U/ml.

RESULTS

Construction of a Recombinant Ectromelia Virus Expressing rZP3

The recombinant virus ECTV-ZP3 was constructed by first infecting tissue cells with ectromelia virus followed by DNA transfection with a vaccinia virus transfer vector containing the ZP3 cDNA (pMJ602-ZP3). DNA recombination between the ectromelia virus *thymidine kinase* (*tk*) gene and the vaccinia virus *tk* gene sequences contained in the transfer vector resulted in the insertion of the mouse ZP3 and *Escherichia coli lac*Z genes into the ectromelia virus *tk* gene. Since the viral *tk* gene is disrupted, the recombinant virus has a thymidine kinase negative phenotype. Expression of rZP3 by ECTV-ZP3 was demonstrated by both indirect immunofluorescence detection of plasma membrane expressed rZP3 (Fig. 1) and by immunoblotting of virus-infected cells (Fig. 2) using IE10 rat monoclonal antibody specific for mouse ZP3 [10].

Clinical Symptoms of Mice Infected with Recombinant Ectromelia Viruses

To study the in vivo effect of the recombinant virus, BALB/c mice were inoculated in the right hind foot-pad with 10^6 pfu of ECTV-ZP3 or a control virus expressing β-galactosidase only (ECTV-602). Mice infected with ECTV-ZP3 or ECTV-602 displayed identical mild symptoms. After 6 days, the inoculated foot became moderately swollen, but this had subsided by 14 days p.i., with all mice recovering from the infection. Infection of BALB/c mice with either 10^3 or 10^6 pfu ECTV-ZP3 by foot-pad inoculation generated similar clinical symptoms and antibody responses towards rZP3 (see below). These symptoms contrast with those seen after infection with the virulent wild-type Moscow strain of ectromelia virus, which results in a systemic infection and high mortality of BALB/c mice [13]. The marked attenuation of the recombinant viruses is probably due to disruption of the *tk* gene, as has been observed previously [17, 28].

Increasing ECTV-ZP3 viral titers were detected in the inoculated feet between Days 1 (average titer 4×10^4 pfu/g tissue) and 9 (6×10^8 pfu/g) p.i. By 13 days p.i., the ECTV-ZP3 infection was resolving (titers ranging between 5×10^2 and 8×10^3 pfu/g). ECTV-ZP3 could not be readily isolated from the inoculated feet at 22 days p.i., when the mice had recovered, although with some mice residual virus was still evident (5×10^2 pfu/g). Virus could not be detected in spleen or ovaries of mice infected with either

ECTV-ZP3 or ECTV-602 from the time of infection to 22 days p.i. This again contrasts with the growth of virulent Moscow strain in BALB/c mice, in which virus could be recovered from the spleen (3×10^8 pfu/g), ovaries (2×10^6 pfu/g), and inoculated foot (7×10^9 pfu/g) at Day 9 p.i., when the animals had either died or were killed.

Infertility of Female Mice after Infection with ECTV-ZP3

Two groups of female BALB/c mice were infected with either 10^6 pfu of ECTV-602 or ECTV-ZP3 and allowed to recover. The female mice were then individually paired with males at 3 wk p.i. to determine their fertility compared to age-matched uninfected controls (Table 1). This mating trial demonstrated a marked reduction in the fertility of the female mice after only a single inoculation of ECTV-ZP3. Nine of 13 mice infected with ECTV-ZP3 failed to conceive, and those that did had fewer implantation scars and smaller litters (average of 1.8 pups) compared to those infected with ECTV-602 (average of 7.3 pups) or untreated animals (average of 6.6 pups).

At 8 wk p.i., ovaries collected from 5 of 13 ECTV-ZP3-infected mice had an abnormal appearance, with a smooth outer surface, and they contained only small (types 1, 2, and 3a) and medium (type 3b) follicles (Fig. 3B). The remaining volume of the ovaries was occupied by large clusters of "luteinized" cells. Ovaries from the remaining infertile and subfertile ECTV-ZP3-challenged mice and mice from both control groups had a normal range of small, medium, and large preovulatory (type 8) follicles (Fig. 3A).

In a further experiment, ovarian follicles appeared normal in mice infected with ECTV-ZP3 at 1, 2, and 3 wk p.i. Changes were apparent in some mice at 4 wk p.i. with the disappearance of large follicles. There was no evidence of a major inflammatory infiltrate between 1 and 6 wk p.i., suggesting that there was no significant cell-mediated response against ZP3 expressed by the recombinant virus.

Although no significant inflammatory response was observed in ovaries of ECTV-ZP3-infected mice, intense antibody binding to the ZP could be detected by direct immunofluorescence (Fig. 3D). Serum collected from mice 2 wk p.i. with ECTV-ZP3 contained detectable levels of anti-rZP3 antibodies, which increased by 7 wk p.i. as determined by immunoblotting (Fig. 4). Indirect immunofluorescence was used to demonstrate that these anti-rZP3 antibodies could further bind to the endogenous ZP of uninfected mice (Fig. 5).

Prolonged Contraceptive Response and Effect of Revaccination with ECTV-ZP3

A longer-term fertility trial demonstrated a prolonged effect of ECTV-ZP3 on fertility. A sustained anti-rZP3 antibody response could be detectable by ELISA for up to 40 wk after the initial infection with ECTV-ZP3 (Fig. 6). Female BALB/c mice were infected with 10^3 pfu of either ECTV-ZP3 or ECTV-602 by foot-pad inoculation and then

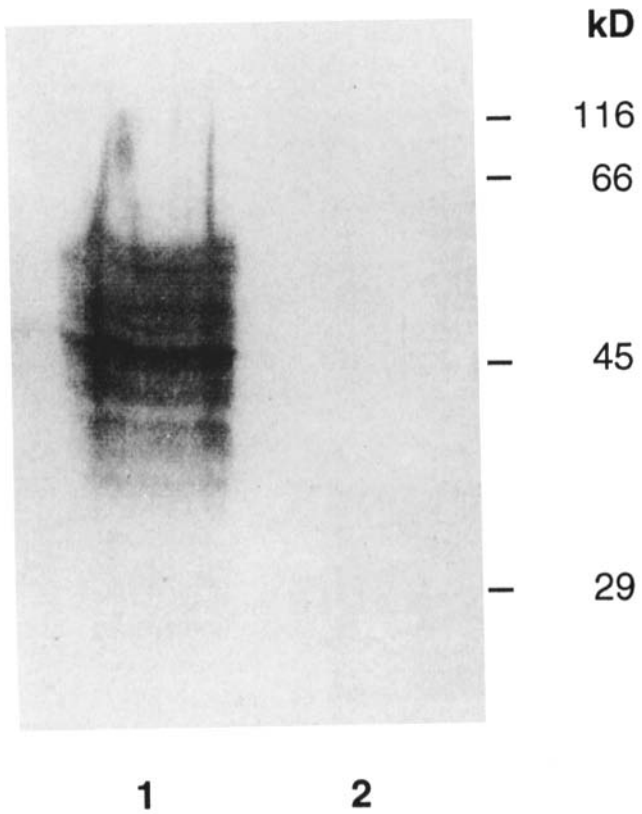

FIG. 2. Immunoblot of ECTV-ZP3-infected tissue culture cells. Immunoblot of LM(TK−) cells infected with ECTV-ZP3 (lane 1) and ECTV-602 (lane 2) probed with monoclonal antibody IE10 rat anti-mouse ZP3. The molecular weights ($\times 10^{-3}$) of Color Markers (Sigma Chemical Company) are indicated on the right.

individually paired with male mice 2 wk p.i. The results of this first breeding trial indicated that 3 of 5 ECTV-ZP3-infected animals were infertile, while the remaining 2 animals were subfertile, producing small litters. A second mating of the subfertile animals 5 wk later failed to produce litters, indicating that complete infertility had been accomplished. One animal that was infertile 2 wk p.i. died during the second mating trial and is not included in Figure 6. The four surviving animals (Fig. 6, B–E) remained infertile for at least 13 wk p.i. One animal (Fig. 6C) had a small litter (2 pups) following the mating at 23 wk p.i., suggesting that it was subfertile at this time. By 37 wk p.i., three of the surviving animals had returned to full fertility, as indicated by the normal litter size (Fig. 6, B, C, and E). The remaining animal was infertile for at least 37 wk p.i.; unfortunately this animal died during the subsequent mating trial (Fig. 6D). The mice that were subfertile at 2 wk p.i. were the first to return to fertility, suggesting that these animals exhibited a lower immunocontraceptive response, and as the circulating antibody levels to the ZP3 auto-antigen waned, they returned to fertility (Fig. 6, B and C).

TABLE 1. Infertility in mice infected with ECTV-ZP3.

Ectromelia virus infection	No. of mice with litters/total mice	No. of implantations (mean ± SEM)		Litter size (mean ± SEM)	
		Animals with litters	All animals	Animals with litters	All animals
None	10/10	9.5 ± 0.8	9.5 ± 0.8	6.6 ± 0.8	6.6 ± 0.8
ECTV-602	12/15	8.5 ± 0.9	6.8 ± 1.1	7.3 ± 0.7	5.8 ± 1.0
ECTV-ZP3	4/13	2.5 ± 0.7	0.8 ± 0.4	1.8 ± 0.3	0.5 ± 0.2

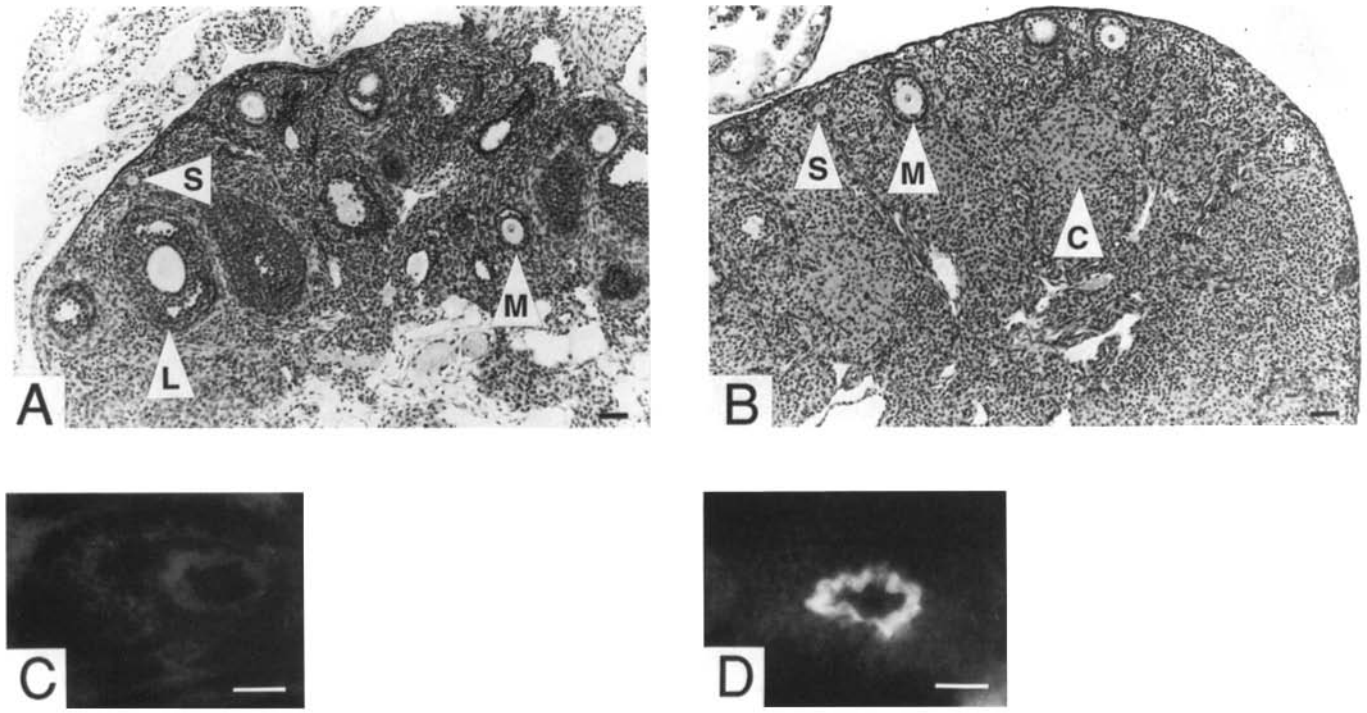

FIG. 3. Histology of ovaries from mice immunized with recombinant ectromelia virus. Ovarian sections from an ECTV-602-infected mouse showing normal follicles (**A**), and a mouse infected with ECTV-ZP3 showing marked depletion of mature follicles (**B**). S, small follicle; M, medium follicle; L, large follicle; C, "luteinized" cell cluster. Direct immunofluorescence of frozen ovarian sections collected from an ECTV-602-infected mouse (**C**) and an ECTV-ZP3-infected mouse showing immunoglobulin bound in situ to the ZP (**D**). Scale bars = 50 μm.

To determine whether the anti-rZP3 immune response could be boosted, the three mice that had recovered fertility (Fig. 6, B, C, and E) were revaccinated by inoculation into the right hind foot-pad with 10^6 pfu of ECTV-ZP3. The infected animals displayed a delayed hypersensitivity response 24–48 h p.i. with mild swelling of the inoculated foot, which returned to normal size over the next few days. Analysis of serum collected 2 wk post-boosting demonstrated that the autoimmune anti-rZP3 response in these animals had returned to maximal levels (Fig. 6). When these animals were subsequently mated, they were again infertile.

DISCUSSION

In this study, we have demonstrated for the first time the feasibility of using a live recombinant virus as an immunocontraceptive vaccine. Mice infected with ECTV-ZP3 developed anti-ZP antibodies that bound in situ to the ovarian ZP. This was accompanied by a prolonged period of infertility of between 5 and 9 mo p.i. As the anti-rZP3 titer in the serum decreased, the animals returned to fertility. However, revaccination of these fertile animals with ECTV-ZP3 resulted in a boosting of the anti-rZP3 response, and the animals were again rendered infertile. It should be noted that the fertility of the age-matched controls was also decreasing, almost a year after the first infection with the recombinant viruses (Fig. 6A).

Antibodies against ZP3 can be sufficient to cause infertility. Antibodies coating the mature oocytes could prevent fertilization by inhibiting sperm-binding to ovulated eggs or penetration of the sperm through the ZP [10]. Anti-zona antibodies are also known to inhibit the shedding of the ZP by the blastocyst before implantation [29]. ECTV-ZP3 may also induce infertility in some mice less directly, by disrupting folliculogenesis. The ovaries of almost half the rZP3 immune mice were structurally abnormal, with large numbers of luteinized cell clusters and the absence of medium and preovulatory follicles. These changes did not appear to result from either a major inflammatory response or from damage due to viral infection of the ovary. The antibodies bound to the developing ZP of growing oocytes

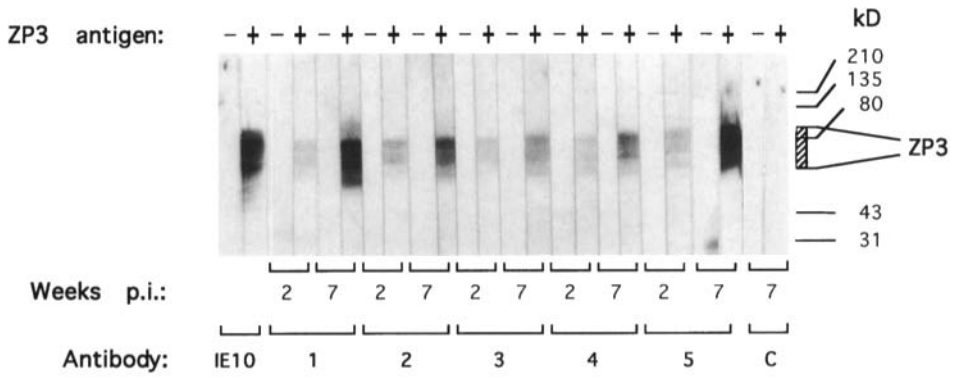

FIG. 4. Immunoblotting detection of anti-rZP3 antibodies in sera of mice infected with ECTV-ZP3. Serum was collected from mice infected with ECTV-ZP3 (1–5) or control ectromelia virus ECTV-602 (C) at 2 and 7 wk p.i and used to probe blots of proteins prepared from cells infected with MYXV-ZP3 expressing rZP3 (+) or wild-type myxoma virus (−). Lanes probed with positive control rat monoclonal antibody anti-mouse ZP3 are shown (IE10). The molecular weights ($\times 10^{-3}$) of Kaleidoscope Prestained Standards (Bio-Rad Laboratories, Hercules, CA) are indicated on the right.

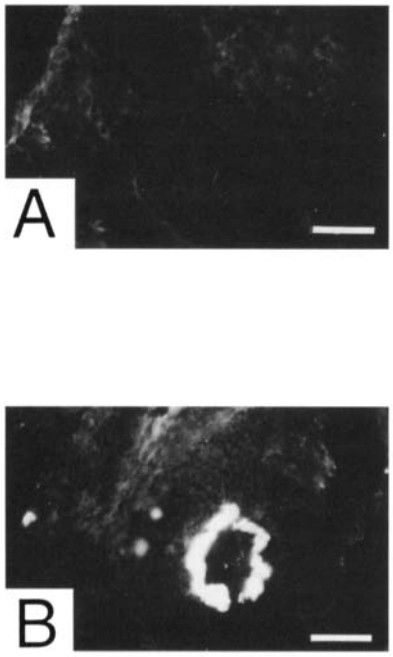

FIG. 5. Indirect immunofluorescence detection of anti-ZP antibodies in the sera of mice infected with ECTV-ZP3. Indirect immunofluorescence of frozen normal ovarian sections probed with serum from an ECTV-602-infected mouse (**A**) and an ECTV-ZP3-infected mouse showing immunoglobulin bound to the ZP (**B**). Scale bars = 50 μm.

[3, 4] may disrupt folliculogenesis, possibly by killing of the oocyte via antibody-dependent cell-mediated cytotoxicity or complement lysis. Alternatively, the attachment of antibodies to the developing ZP could interfere with normal follicular development, which relies on close communication between the oocyte and its surrounding granulosa cells [30]. Disruption to follicular development has been observed in connexin 37-deficient mice, in which the failure to form gap junctions between the oocyte and surrounding granulosa cells prevents intercellular signaling, resulting in the premature luteinization of the granulosa cells and inappropriate corpus luteum formation [31].

Immunization of certain inbred mouse strains with a ZP3 peptide (amino acid residues 330–342) stimulates autoimmune antibody in association with CD4+ T cell-mediated ovarian inflammation (oophoritis) [12, 32]. However, ZP3 peptide vaccine-induced oophoritis appears to be mouse strain-dependent: oophoritis was not observed in other inbred strains and outbred mice after immunization [11, 12]. We also found no evidence of ovarian inflammation in ECTV-ZP3-infected BALB/c mice. Interestingly, immune cell infiltration is not observed in rabbits immunized with total pig ZP [33] or in monkeys immunized with recombinant rabbit ZP proteins [34], in which the ovarian pathology appears to be mediated by antibody specific to the ZP.

The strong immune response against ZP3 in ECTV-ZP3-infected mice is unusual, since adult mice generally display immune tolerance to "self" antigens. However, previous studies have demonstrated that antibody to ZP3 can be induced by immunization with ZP3 peptides [11, 12]. These studies suggest that ZP3-reactive B cells are present (but inactive) in nonimmunized female mice and may be primed by endogenous ZP antigens released during follicular atresia [35, 36]. The failure of these B cells to differentiate and produce antibody may be due to the lack of appropriate T-cell help. In our experiments, this T-cell help could have been provided through recognition of viral epitopes. ZP3-specific B cells might be infected by virus or could take up

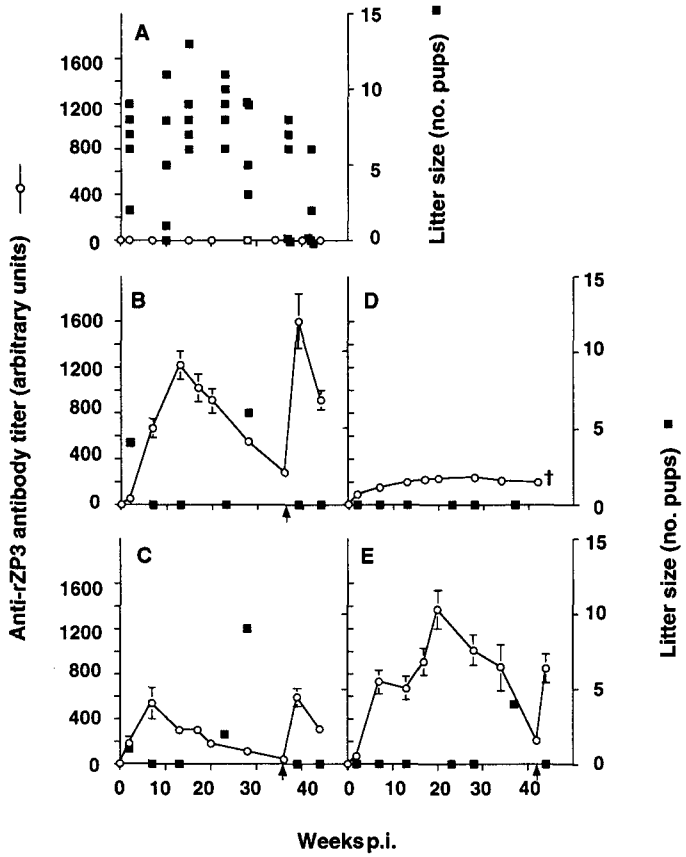

FIG. 6. Time-course of ZP3 immune response and duration of infertility in mice infected with ECTV-ZP3. Two groups of 5 female BALB/c mice were infected by foot-pad inoculation with 10^3 pfu of either control virus ECTV-602 (**A**, pooled results for 5 mice) or ECTV-ZP3 (**B–E**, individual mice; one mouse died 8 wk p.i. and is not included), were allowed to recover, and then were periodically mated with males to determine their fertility. Anti-rZP3 antibody titers (mean ± SD) detected by ELISA, and litter size are plotted against weeks p.i. Arrows (**B, C, E**) indicate the time of revaccination with ECTV-ZP3. A cross in **D** indicates the death of this animal.

membrane fragments released from virus-infected cells containing ZP3 antigen complexed with viral proteins. These B cells would present both ZP3 and viral peptides on their surface and could be stimulated by virus-specific T helper cells to differentiate and secrete ZP3 specific antibody. Alternatively, T-cell tolerance may have been broken as a result of enhanced levels of antigen presentation or altered posttranslational processing of ZP3 by the virus-infected cells, or because of cytokines produced in the immune response to viral antigens.

The role of endogenous ZP antigens in the immune response to ZP3 expressed by ectromelia virus is not clear. The return to fertility suggests that endogenous ZP is incapable of maintaining the immune response generated by infection with ECTV-ZP3. Furthermore, it appears that endogenous antigen is not necessary for the immune response since in preliminary experiments, mice ovariectomized before infection developed anti-ZP3 antibody levels similar to those of infected sham-operated mice (unpublished results). Nevertheless, endogenous ZP antigens could play an important role in broadening the specificity of the antibody response to components of the ZP other than ZP3, by the phenomenon of epitope spreading [35, 36]. A B cell specific for ZP2, for example, could take up and process fragments of ZP containing both ZP3 and ZP2. Activated ZP3-specific T helper cells could then stimulate anti-ZP2 antibody production by this B cell. Whether this occurs in ECTV-ZP3-infected mice remains to be investigated.

Although we have chosen the well-characterized ZP3 antigen, there are several other gamete antigens (LDH-C4 [37], PH20 [38], p95 [39], and SP17 [40]) and reproductive hormones (GnRH [41], FSH [42], and chorionic gonadotropin [43]) that could be included in a suitable vector. Indeed, recombinant viruses could be constructed that express more than one fertility-associated antigen. Such viruses could be constructed to maintain the wild-type disseminating phenotype by insertion of the reproductive antigen genes into noncoding regions [22, 44], or alternatively by inactivation of nonessential virulence genes to generate nondisseminating attenuated vectors.

The acceptability of this controversial approach to population control [45, 46] will depend on a number of factors. Species specificity of the immunocontraceptive vaccine will be an important consideration. Any disseminating viral vector would ideally infect only the target species. A nondisseminating immunocontraceptive agent could be delivered through bait. For example, in Europe the control of rabies in foxes was achieved through oral bait vaccination using live attenuated rabies vaccine or, more recently, a recombinant vaccinia virus expressing the rabies G protein [47]. We wish to stress, however, that extensive investigations of potential risks and benefits are essential before any fertility control agent could be introduced into the environment.

ACKNOWLEDGMENTS

We thank Dr. Jurrien Dean (Laboratory for Cellular and Developmental Biology, NIH Bethesda, MD) for providing pZP3.4 and IE10 antibody, and Dr. Michael Peek for assistance with the histology. We also thank J. Grigg, K. Groom, D. Hall, N. Siddon, V. Quinn, K. White, and J. Wright, for technical assistance.

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