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Immunoglobulin to zona pellucida 3 mediates ovarian damage and infertility after contraceptive vaccination in mice

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ABSTRACT

Antibodies reactive with the ovarian glycoprotein zona pellucida (ZP) have been linked with human female infertility. Anti-fertility vaccines that target ZP antigens have been utilized to restrict pest animal populations and their efficacy is associated with ovary-specific antibody induction. However, the necessity for zona pellucida-specific antibody in mediating infertility has not been examined *in vivo*. A recombinant mouse cytomegalovirus vaccine encoding murine zona pellucida 3 that induces rapid and complete infertility in BALB/c mice has been produced. The onset of infertility is temporally related to the presence of antibody sequestered into ovarian follicles and binding to the ZP of infected mice and the loss of mature follicles. When this vaccine was inoculated into immunoglobulin-deficient BALB/c mice with a null mutation in the immunoglobulin mu chain gene *Igh-6*, fertility was unaffected. Passive transfer of serum containing ZP3 antibodies also elicited transient infertility. Electron microscopy of ovarian tissue collected from ZP3-immunized immunocompetent mice demonstrated significant focal thinning of the zona pellucida (ZP) with reduced length and concentration of transzonal processes and many oocytes displayed evidence of injury. None of these changes were found in vaccinated immunoglobulin-deficient mice. These data confirm that ZP3-reactive antibody is necessary and sufficient to induce autoimmune-mediated follicular depletion and fertility suppression following the inoculation of this vaccine, and suggest that this is due to impaired zona pellucida formation. These findings have relevance in understanding the etiology of autoimmune ovarian disease in woman where anti-ZP antibodies are likely to have a causal role in infertility.

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1. Introduction

Immunocontraception is a process where targeted autoimmunity is induced towards a self antigen that is crucial to fertilization of the oocyte and/or pregnancy progression. This leads to a reduction in or cessation of fertility [1]. This approach has been investigated as a potential technology to control various large animal populations, but with mixed success [2–5]. The association between a strong antibody response and successful fertility suppression has been well established and the magnitude of the antibody response induced by potential immunocontraceptive antigens correlates with contraceptive efficacy [6,7]. However, since infertility was usually incomplete and temporary in these studies, it was difficult to elucidate the precise role of antibody in suppressing reproduction.

A recombinant murine cytomegalovirus expressing the murine follicular antigen zona pellucida 3 (rMCMV-mZP3) has been shown to induce long-lasting infertility in BALB/c mice after a single inoculation [8]. This infertility effect has largely been attributed to the production of ZP3-specific antibody, which increases over time in the sera of infected mice and accumulates within the ovaries of infected mice *in vivo*. However, no direct causal link has been demonstrated between ZP3 antibody and infertility.

A model of mouse oophoritis in which ZP3 peptides are inoculated in Complete Freund's adjuvant into (C57BL/6 × A/J) F1 mice [9] demonstrated that the presence of ZP-specific antibody redirected cellular immunological damage from atretic follicles (in the absence of antibody) to developing and mature follicles, with subsequent ovarian atrophy and follicular loss [10]. In comparison, the inoculation of mice with rMCMV-mZP3 resulted in an influx of inflammatory leukocytes (predominantly CD4+ and CD8+ T cells)

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into the ovarian tissue [11]. The inflammatory infiltrate was present throughout the ovarian tissue in developing follicles, corpora lutea and the ovarian stroma [11]. However, there was no evidence of overt inflammatory lesions in ovaries from rMCMV-mZP3 infected mice even though ZP antibodies were produced [8]. Knowing that T cell inflammation was present, we wanted to determine whether antibody production was essential for successful immunocontraception and to understand the pathophysiological consequences of autoantibody production for the follicles of infected mice.

We describe here the absolute requirement for ZP3-specific antibody for immunocontraception to be successful after inoculation with rMCMV-mZP3. Vaccinated immunoglobulin-deficient mice did not become infertile and their ovaries appeared histologically normal, but passive transfer of ZP3-specific antibody into naïve mice induced infertility. This result demonstrates the paramount role of the antibody response in mediating immunocontraception and indicates a likely pathophysiological action of anti-ZP antibody in the ovarian function of women with unexplained infertility.

2. Materials and methods

2.1. Mice

Except where otherwise indicated, BALB/cArc (BALB/c) mice were obtained as certified specific pathogen free (SPF) from the Animal Resources Centre (Murdoch University, Perth, Western Australia) and were maintained under barrier conditions in filter top cages. BALB/c mice were also imported from the National Cancer Institute (Bethesda, MD, USA) for one experiment (BALB/cNIH). All BALB/c mice were 8 weeks old when inoculated. Immunoglobulin-deficient mice with a neomycin disruption of a membrane exon of the immunoglobulin mu chain gene, *Igh-6^{tm1Cgn}*, on a BALB/c genetic background, henceforth referred to as μMT mice, were kindly provided by Professor Lynda Morrison (St Louis University, Mo, USA). The μMT mice were used in experiments at 6–15 weeks of age and were bred at the University of Western Australia animal breeding facility. The production of these mice has been previously described [12]. The homozygous neomycin gene insertion was confirmed by PCR of genomic DNA extracted from tail tips, and the absence of antibody was confirmed by both ELISA and an immunoblot of serum on nitrocellulose (results not shown). On completion of the individual experiments, all mice were euthanized by methoxyflurane inhalation (Penthrox, Medical Developments International Ltd., Australia) followed by cervical dislocation. The presence of known murine pathogens including MCMV was excluded by regular testing of sentinel mice. Because many tests for murine pathogens are serological, sentinel mice were originally held in the same cage as μMT mice to confirm the absence of any pathogens. Mouse care was based on the Australian Code of Practice endorsed by the National Health and Medical Research Council, and was approved by the University of Western Australia's Animal Ethics Committee.

2.2. Virus

The production and characterization of the recombinant viruses, rMCMV-mZP3 and rMCMV-Ova (recombinant murine cytomegalovirus expressing mouse ZP3 and recombinant murine cytomegalovirus expressing chicken egg ovalbumin), have previously been described [8,11]. The antigens were cloned into the immediate early 2 region of the virus and the recombinant viruses demonstrated equivalent *in vitro* growth to parental virus strains [8]. The MCMV control virus used in the long term breeding experiments was K181 (Perth), a laboratory passaged strain that has been previously described [13]. Virus stocks were maintained on mouse embryo fibroblast (MEF) cells and were regularly titrated to confirm viral titer. All virus inocula were 2×10^4 plaque forming units (pfu)/mouse and were inoculated intraperitoneally (i.p.).

2.3. Plaque assay

Virus stocks were diluted in MEM + 2% NCS starting at 1:10 and continuing in 1:4 dilutions. Twenty-four well cell culture trays with 95% confluent MEFS were inoculated with 200 μL per well of each dilution. After 1 h incubation in 5% CO_2, 37 °C the suspension was removed and a layer of 0.5% methylcellulose/MEM + 2% NCS was applied. The plates were incubated for 4 days at 37 °C, 5% CO_2 and stained with 1 ml 0.05% methylene blue with 10% formaldehyde per well overnight. After washing, the number of plaques in each well was enumerated, and the dilution was used to calculate the plaque forming units per ml.

2.4. Hyperimmune sera

Hyperimmune serum (HIS) was generated by three intraperitoneal inoculations at two-week intervals of BALB/c mice with 2×10^4 pfu of rMCMV-ZP3 or rMCMV-Ova. Serum was collected two weeks after the final inoculation and is referred to as rMCMV-mZP3 HIS or rMCMV-Ova HIS.

2.5. Monoclonal antibody

The rat ZP3 1gG2a monoclonal antibody ATCC CRL-2462 has been previously described [14]. The IE10 hybridoma was stored in liquid nitrogen and was propagated in Dulbecco's MEM (Invitrogen, Auckland, NZ) supplemented with 20% FCS, 10 mM HEPES (Invitrogen, Aukland, NZ) and 0.05 mM 2-mercaptoethanol at 37 °C, 5% CO_2. When confluent, the hybridoma cells were harvested, resuspended in serum-free medium and cultured until cell death. The culture medium was recovered and clarified at 480× g for 10 min and the ZP3 antibody was purified by the addition of saturated ammonium sulphate. The antibody was centrifuged at 5000× g and the precipitate resuspended in PBS, dialyzed against PBS overnight and sterilized by filtration through a 0.2 μM filter. The monoclonal antibody was stored at −80 °C. The concentration of monoclonal antibody was estimated using the Biorad protein assay (Biorad Laboratories, CA, USA).

2.6. Breeding studies

Female BALB/c mice were inoculated i.p. with 2×10^4 pfu virus or were sham-inoculated (100 μL PBS diluent) and male mice were introduced at a ratio of 1 male:1 female immediately post-inoculation. The breeding output (number of pups born) over 100 days was recorded. There was no compensation made for pups eaten or stillborn. Each female produced one litter before the immunocontraceptive effect became evident and this allowed the fertility of the mice to be confirmed within the experiment.

In a second experiment female BALB/c mice were inoculated intravenously with 100 μL hyperimmune serum or ZP3 monoclonal antibody, IE10. Twenty-four hours later, male mice were introduced at a ratio of 1 male:2 females. Mice were maintained together until pregnancy was established. The time from the introduction of the male to the birth of the first litter was recorded.

Separately, female μMT mice were inoculated with 100 μL monoclonal antibody intravenously; one group was inoculated with IE10 and another control group was inoculated with 100 μL sterile PBS. Male mice were introduced one week later at a ratio of 1 male:1 female. Mice were maintained together until pregnancy was established. The time from the introduction of the male to the birth of the first litter was recorded. Additionally, female μMT mice

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