Antibody Responses and Infertility in Mice Following Oral Immunization with Attenuated Salmonella typhimurium Expressing Recombinant Murine ZP3

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ABSTRACT

Ovarian ZP3, the primary sperm receptor, is a major glycoprotein of mouse zona pellucida (ZP). Because antibodies raised against ZP3 block sperm-egg interaction, ZP3 has been considered a candidate immunogen in the development of a contraceptive vaccine. This study explored the possibility of using an attenuated Salmonella typhimurium vaccine strain expressing recombinant ZP3 to elicit an antibody response and infertility in mice. A cDNA sequence generated by the polymerase chain reaction encoding 342 amino acid residues (23-364) of the mouse (m)ZP3 was cloned into an Asd+ vector. An avirulent Salmonella vaccine strain stably expressed the ZP3 polypeptide and colonized the internal organs of mice after oral inoculation. Oral immunization of female BALB/c mice with the recombinant Salmonella vaccine strain expressing mZP3 induced significant levels of anti-native ZP IgG antibodies in serum and IgA antibodies in vaginal secretions. The IgG antibodies thus induced also bound to ZP in vivo. When mated with males, 3 of 6 females immunized with the recombinant Salmonella were infertile. In contrast, none of the mice that received Salmonella containing the vector plasmid produced antibodies to ZP and all were fertile. No ovarian inflammation was observed in the immunized mice at autopsy. The results suggest a potential oral contraceptive vaccine to control populations of rodent vectors of disease and to induce reversible infertility in humans.

INTRODUCTION

Immucontraception has received increased interest in past decades as a potential way of controlling the rapid growth of world population [1]. The induction of immune responses against gamete-specific antigens represents one approach to developing anti-fertility vaccines [2]. Gamete-specific antigens to be used for developing a contraceptive vaccine must be the functional molecules involved in the process of fertilization, or gamete development and transportation. A number of specific antigens, including zona pellucida (ZP) proteins, have been identified [3-5].

ZP is an extracellular matrix surrounding the developing and mature ovum. In the mouse, ZP is composed of three major glycoproteins, ZP1, ZP2, and ZP3 [6]. ZP3 serves as primary sperm receptor mainly through its O-linked oligosaccharide [7]. Monoclonal antibodies (mAb) raised against ZP3 effectively inhibit fertilization both in vivo and in vitro [8]. In several mammalian species, immunization with purified heterologous ZP antigens has been shown to inhibit or reduce fertility in the immunized animals, probably through function of anti-ZP antibody [9-11]. In developing a contraceptive vaccine, it is important to ask whether native ZP antigens can be replaced by a recombinant protein or synthetic peptide. Since ZP proteins are highly glycosylated and naturally insoluble, the immunogenicity of the recombinant protein, especially expressed in prokaryotic cells, could be quite different from that of the native form. A number of findings, however, support the view that antibodies to recombinant ZP protein may still exhibit contraceptive activity. For instance, it has been shown that antibodies directed against the polypeptide backbone of ZP protein can interfere with sperm binding to or penetration of ZP [12]. Immunization with fully or partially deglycosylated ZP proteins has resulted in reduced fertility in some species [13]. A linear B epitope of ZP3 has been mapped, and antibodies to synthetic peptides containing this region recognize native ZP and inhibit fertilization in vivo [14, 15].

An attenuated vaccine strain of Salmonella typhimurium with deletions of the adenylate cyclase (cya) and cAMP receptor protein (crp) genes is avirulent but retains its ability to colonize the gut-associated lymphoid tissue and internal organs [16]. In addition, Salmonella is an excellent host for expressing recombinant proteins. It has been shown that oral immunization with attenuated Salmonella expressing foreign antigens stimulates antigen-specific secretory, humoral, and cellular immune responses (for review see [17]). Very recently, a recombinant Salmonella strain expressing a human sperm-specific antigen, SP-10, successfully induced systemic as well as mucosal antibody responses in the murine female reproductive tract following oral immunizations [18], representing a novel way of delivering contraceptive vaccine.

In the present study, we explored the possibility of inducing anti-ZP3 antibodies in mice following oral immunization of mice with attenuated Salmonella expressing recombinant mouse (m)ZP3. A partial-length cdNA encoding amino acid sequence 23-364 of murine ZP3 was cloned into the l-aspartate semialdehyde dehydrogenase (asd)-based vector, which is stable for prolonged expression in vivo [19, 20]. Oral immunization of mice with the S. typhimurium strain containing the Asd+ construct elicits both systemic and mucosal antibody responses to native ZP.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The attenuated S. typhimurium ΔcrpΔcyaΔasd strain χ4550 was derived from χ4064 [16]. Escherichia coli χ6212 is an asd deletion mutant of E. coli DH5α containing the lacZ-α gene (Bethesda Res. Lab. Life Technol., Gaithersburg, MD) [21], and was used as an intermediate transformation recipient of plasmid DNA. Luria broth (LB) supplemented with 50 μg/ml of DL-α,ε-diaminopimelic acid...
(DAP) from Sigma Chemical Company (St. Louis, MO) was used for growing bacterial strains with the asd deletion mutation. LB agar plates or MacConkey agar plates supplemented with 1% maltose were used for determination of colony-forming units (CFU).

Construction of Plasmid Vectors

*Salmonella* expression vectors pYA3137 and pYA3149 are derivatives of the pU1A-based low copy number plasmid pYA292 [19]. Both vectors contain the trc promoter, multiple cloning sites, and the asd gene. Plasmid pYA3137 has the pUC18 origin of replication and is present in 200–400 copies per chromosome DNA equivalent, while pYA3149 has the pBR322 origin of replication and is present in 50–100 copies per chromosome DNA equivalent. All initial cloning of the recombinant plasmid was carried out in *E. coli* strain χ6212.

The cDNA-encoding mZP3 gene was originally cloned by Ringuette et al. [22]. Plasmid pBluescript KS containing the cDNA sequence of mZP3 (kindly provided by Dr. P. Saling, Duke University, Durham, NC) was used as a polymerase chain reaction (PCR) template. Amplification of mZP3 gene encoding amino acids (aa) 23–424 was conducted using the two primers, 5’ gggaagcctTTATTGCGGAAGGGA GGCTTTTGCCG 3’ (nucleotides 96–116) containing an *EcoRI* site and 5’ gggaagcttTTATTGCGGAAGGGA TACAAG 3’ (nucleotides 1283–1304) containing a *HindIII* site. Approximately 10 ng of pBluescript-mZP3 plasmid DNA was used in the PCR under the following conditions: 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C for 30 cycles. The 1227-base pair (bp) PCR product, which corresponded to the expected size, was purified by Geneclean (Bio101, La Jolla, CA) and cloned into the vectors.

**Construction of Recombinant Salmonella Expressing mZP3**

The recombinant plasmid containing mZP3 gene was electroporated into χ6212, and electroporants were selected by using MacConkey agar plates without DAP added. Ten colonies were tested for the insert and correct plasmid size. The plasmid was then purified from χ6212 and electroporated into *S. typhimurium* χ4550.

**Characterization of Salmonella Expressing mZP3**

To detect expression of mZP3, *S. typhimurium* strains harboring a plasmid with or without mZP3 cDNA were grown with aeration overnight at 37°C. Five hundred microliters of culture was centrifuged (10,000 rpm, 10 min, 4°C), and pelleted cells were lysed in SDS-PAGE loading buffer. The proteins present in the whole cell lysate were separated by SDS-PAGE using 12.5% gels. Gels were prepared for either Coomassie brilliant blue staining or Western blot analysis whereby the separated proteins were electrotransferred to nitrocellulose membrane. The membrane was then blocked in a Tris-HCl buffer (pH 7.4) solution containing 2% BSA and 0.1% Tween 20 (Sigma) and incubated with a 1:50 dilution of the rat mAb IE-10 (kindly provided by Dr. P. Saling, Duke University, Durham, NC), which recognizes ZP3 peptide aa 326 to 342 [13]. The membrane was further incubated with a goat anti-rat IgG antibody conjugated with alkaline phosphatase. The reactive protein bands were visualized using a mixture of nitroblue tetrazolium (0.37 mM) and 5-bromo-4-chloro-3-indolyolphosphate (0.34 mM) in 100 mM Tris-HCl buffer with 100 mM NaCl and 5 mM MgCl₂, pH 9.5.

To test plasmid stability in vitro, 5 ml of LB was initially inoculated with plasmid-containing *S. typhimurium* from a frozen stock and grown with aeration overnight at 37°C. Ten microliters of the overnight culture was inoculated into tubes containing 5 ml of LB with DAP and the tubes were cultured without aeration. This procedure was repeated four times (approximately 50 generations), and serial dilutions of each subculture were plated on LB plates with DAP. Twenty hours later, the plates were replicated onto plates without DAP. Colonies that grew on the plates without DAP were considered Asd⁻, and the rates of maintenance of the asd containing plasmids were determined.

Growth characteristics of *Salmonella* vaccine strains containing the constructs were determined by inoculating 50 ml LB with 1 ml standing overnight cultures; the cultures were grown with aeration at 37°C. Samples were taken at different time intervals and plated onto LB plates, which were incubated at 37°C. Twenty hours later, colonies were counted and growth curves were determined.

To test the colonization ability of the recombinant *Salmonella* strains, mice were orally inoculated with 10⁶ CFU of bacteria. Peyer’s patches and spleens were removed from the mice on Days 4 and 10 after inoculation and were homogenized in buffered saline with gelatin (BSG) [22]. Samples were plated onto MacConkey agar plates (without DAP) containing 1% maltose and incubated at 37°C for 20 h; the resulting colonies were then counted. The colonies were also selected at random and analyzed for the expression of mZP3 and in vivo stability of recombinant plasmid by Western blot.

**Mice and Oral Immunization**

Four-week-old female BALB/c mice were purchased from Sasco (Omaha, NE) and were housed in autoclavable micro-isolator cages with free access to standard laboratory food and water for 1 wk to acclimate before use. Inocula for oral immunizations were prepared from log-phase cultures of recombinant *S. typhimurium* [16]. Before immunization, mice were deprived of food and water for 4 h and then fed 30 μl of 10% (w:v) sodium bicarbonate solution (pipetted into the back of the mouth with a micropipette) to neutralize stomach acidity. Thirty minutes later, 25 μl sterile BSG, or BSG containing 10⁵ CFU of recombinant *S. typhimurium*, was administrated orally by micropipette. Food and water were returned 30 min later.

**Immunization and Sampling Schedules**

*S. typhimurium* χ4550 (pYA3229), which expressed the truncated mZP3 (aa 23–364) and was capable of colonizing mice, was used to immunize a group of six 5-wk-old female BALB/c mice to determine antibody response. Two other groups were immunized orally with either χ4550 (pYA3149) or sterile BSG and served as negative controls. Initially, mice were given 10⁵ CFU of the bacteria peroral at Day 0 and boosted at 2 mo and 3 mo. Sera and vaginal washings were collected at 5 and 7 wk after the initial immunization and at 2, 4, and 6 wk after the second booster immunization (i.e., 14, 16, and 18 wk after the initial immunization) to measure anti-ZP3 antibodies. A total of five collections were made for the analysis of induction of an antibody response. Ovaries were taken 5 wk after the first immunization and 8 wk after the last immunization to ex-
amine ovarian pathology and to test the ability of anti-ZP3 antibodies to bind to oocytes.

**Collection of Sera and Female Reproductive Tract Secretions**

Immunized mice were anesthetized with Metofane (Pitman-Moore, Mundelein, IL), and blood was collected from the retro-orbital sinus. Serum was separated by standard procedures and stored individually at -70°C until use. Mice were bled prior to immunization (preimmune) as a control. All serum was assayed for the presence of antibody to native ZP. Vaginal secretions were collected by repeatedly pipetting (5–6 times) 50 μl of sterile PBS (pH 7.4) into the vaginal opening. The fluid and mucus were mixed vigorously with a vortex mixer and spun briefly in a microfuge. The supernatant fluid was stored at -20°C until the time for assay. Preimmune mucosal secretions were also collected from mice in a similar manner for use as negative controls.

**Detecting Antibody to ZP by ELISA and Immunofluorescence**

Both procedures used for detection of antibody have been described elsewhere [23]. Briefly, each well of a 96-well flat-bottomed plate (Corning, NY) was coated with 100 ng solubilized mouse ZP in 50 μl of coating buffer overnight at 4°C and blocked by 3% BSA in PBS (pH 7.4) to avoid nonspecific binding. Serum or vaginal washings from experimental mice, nonimmunized control mice, and control mice immunized with vector alone were diluted 1:100 or 1:3, respectively, in PBS with 3% BSA in PBS, and added to each well in duplicate. After the plate was thoroughly washed, it was incubated with peroxidase-conjugated goat anti-mouse IgG (1:3000; Southern Biotech. Assoc., Birmingham, AL) for serum samples or with biotin-conjugated goat anti-mouse IgA (1:5000; Southern Biotech. Assoc.) for vaginal washings. For the biotin-conjugated reagent, the plate was further incubated with avidin biotin-peroxidase complex (Vector, Burlingame, CA). After the final incubation, a substrate mixture of O-phenylenediamine and hydrogen peroxide was added, and absorbance at 490 nm was determined using an ELISA reader (Molecular Devices, Menlo Park, CA).

For immunofluorescence, ovaries from experimental or control animals were snap-frozen in liquid nitrogen and embedded in O.C.T. compound. Sections 5 μm thick were cut with a cryostat. After fixation in 90% ethanol for 15 min, sections were rinsed in PBS. The sections were then incubated with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Capple Inc., Malvern, PA) at a 1:50 dilution in PBS containing 3% BSA and were mounted in glycerol with 10% PBS.

**Mouse Fertility Trial**

To test whether immunization of mice with *Salmonella* vaccine expressing ZP3 can induce infertility, another group of mice were immunized and placed into a fertility trial. Six-week-old female BALB/c mice, six in each group, were orally immunized with 10⁹ CFU of either χ5505 (pYA3229, mZP3 aa 23–364) or χ5505(pYA3149, vector control) or with PBS. Six weeks later, nonimmunized male C3He mice were introduced to females and allowed to mate with them for at least four ovulation cycles (16 days). One male was housed with three females, and the male was rotated once a day to a new group of three females. Pregnant female mice were moved to separate cages, and the date of birth and number of pups born were noted. Four weeks after all the pregnant females had given birth, mice were given two booster immunizations, 1 wk apart, with 10⁹ CFU of the same *Salmonella* strains. Six weeks later, males were again introduced to females and the same mating schedule as described above was followed. For both mating experiments, serum samples were collected from the female mice 1 day before male mice were introduced in order to monitor anti-ZP antibody titers. After the fertility trial, ovaries were removed from the female mice and tested for oophoritis.

**Histologic Assessment of Ovarian Disease**

Ovaries taken from immunized and control mice were fixed in Bouin’s fixative for 24 h and embedded in paraffin. Approximately 50 serial-step sections (5 μm) stained with hematoxylin and eosin were examined. Ovarian pathology was graded, in a double-blind manner, according to increasing severity from 1 to 4: 1, focal inflammation in space; 2 and 3, increasing multifocal inflammatory foci and/or granuloma between and within ovarian follicles; 4, loss of ovarian follicles and ovarian atrophy.

**Statistical Analysis**

Student’s t-test and chi-square test were used to compare differences in pregnancy rate and average litter size between experimental and control animals in the fertility trial and the level of significance in elevation of anti-ZP antibodies.

**RESULTS**

**Cloning and Expression of Murine ZP3 cDNA in S. typhimurium**

Plasmid pBluescript KS containing cDNA encoding the mZP3 gene was used as a PCR template. Two primers for PCR, as described in Materials and Methods, were designed to amplify the mZP3 open reading frame. The first 22 aa were deleted from the N-terminal end of the mZP3 gene to remove the hydrophobic signal sequence that is cleaved during posttranslation processing to give rise to the mature protein [21]. The resultant 1227-bp fragment of PCR product was purified and cloned into analogous sites in pYA3137, an Asd+ vector with a pUC18 origin of replication, to form pYA3201 (Fig. 1); pYA3201 expresses mZP3 aa 23 to 424 with 4 additional amino acids (Met, Pro, Glu, and Phe) added on the N-terminal because of the vector-specifying amino acids. Because the C-terminal end of mZP3 is highly hydrophobic, a characteristic that may negatively affect expression of the protein [24], 180 bp of cDNA encoding 60 amino acids at the C-terminal end were deleted by digestion with Ban II and HindIII. The 5' and 3' overhanging ends of the linearized plasmid were blunt-ended with T4 DNA polymerase and self-ligated to form pYA3202, which expresses a truncated mZP3 protein, aa 23 to 364. The truncated mZP3 gene was then released from the plasmid by EcoRI and HindIII double digestion and ligated into pYA3149, an intermediate copy number Asd+ vector with a pBR322 origin of replication, yielding pYA3229.

The plasmids pYA3201, pYA3202, and pYA3229 were introduced into the S. typhimurium strain χ4550 (ΔcrpΔacya- Δasd). Whole cell lysates of χ4550(pYA3201), χ4550
FIG. 1. Construction of Asd+ vectors specifying mZP3. The open reading frame of mZP3 was amplified by PCR and cloned into a high copy number Asd+ vector, yielding pYA3201. Sixty amino acids were deleted from the C-terminal end of mZP3 by digestion of pYA3201 with Ban II and HindIII, and the remaining plasmid was treated with T4 polymerase and religated, yielding pYA3202. The truncated mZP3 gene was removed from pYA3202 by using EcoRI and HindIII and was ligated into the intermediate copy number asd vector pYA3149 to produce pYA3229.

(pYA3202), and χ4550(pYA3229) were subjected to SDS-PAGE, and the separated proteins were transferred onto nitrocellulose membranes and probed with the mAb IE-10, which recognizes the amino acid sequence 326-342 of native murine ZP3. Two major immunoreactive bands were observed, the 44-kDa band representing the recombinant mZP3 aa 23 to 424 (Fig. 2, lane e) and the 37.8 kDa band representing the truncated mZP3 protein, aa 23 to 364 (Fig. 2, lanes f and g). One lower band of ~35 kDa (in lane e) and two lower bands of 21.5 kDa and 32 kDa (in lane f) were also visible. Presumably the lower molecular mass bands were truncated forms resulting from breakdown of product or interrupted translation. Expression levels of the recombinant mZP3 proteins produced by the two different copy number Asd+ vectors appeared to be different. S. typhimurium containing the high copy number plasmid pYA3229, as judged by the intensity of immunostaining (Fig. 2).

In Vivo and In Vitro Characterization of Vaccine Strains Expressing mZP3

The in vitro growth characteristics of the vaccine strain containing mZP3 constructs were examined and compared to those of the strain containing the Asd+ vector alone. When grown in LB, S. typhimurium χ4550(pYA3201) and χ4550(pYA3202) had similar growth rates; however, both grew more slowly than χ4550 with the vector pYA3137. χ4550(pYA3229) grew equally as well as χ4550(pYA3149) and slightly faster than χ4550(pYA3137).

To determine the in vitro stability of the various Asd+ constructs, vaccine strains were cultivated in LB with DAP.
SALMONELLA VACCINE INDUCES ANTI-ZP Ab AND INFERTILITY

FIG. 2. Expression of mouse ZP3 by Salmonella vaccine strain and antigenicity of the recombinant ZP3 protein. Bacterial cells were grown overnight at 37°C. Five-hundred-microliter cultures were centrifuged, and the cell pellets were resuspended in 50 μl of SDS-PAGE sample buffer and boiled for 5 min. Five-microliter protein preparations from each strain were loaded onto a 12% SDS gel. The separated proteins were either stained by Coomassie brilliant blue or transferred onto a nitrocellulose membrane. The membranes were incubated with rat mAb IE-10, which recognizes aa 336 to 342 of mZP3. Lanes: a, S. typhimurium X4550; b and c, χ4550(pYA3137); d, χ4550(pYA3149); e, χ4550(pYA3201); f, χ4550(pYA3229); g, χ4550(pYA3229).

for 50 generations. When serial dilutions of the subcultures were plated on LB plates with DAP and replicated onto plates without DAP, it was observed that plasmids pYA3149 (Asd+ vector with pBR322 origin of replication) and pYA3229 (mZP3 cDNA cloned into pYA3149) were stably maintained for at least 50 generations. Plasmids pYA3137 (Asd+ vector with pUC18 origin of replication) and pYA3201 (mZP3 cDNA cloned into pYA3137) were gradually lost from the population. This is supported by the fact that the in vitro growth of S. typhimurium containing pUC18-based Asd+ vector was slightly retarded in its growth as compared to S. typhimurium containing pBR322-based Asd+ vector.

The abilities of S. typhimurium vaccine strains harboring pYA3202 and pYA3229 to colonize mice after oral inoculation were investigated. Groups of three female BALB/c mice (5 wk old) were inoculated orally with 1 × 10⁶ CFU of S. typhimurium containing either pYA3202, pYA3229, or vector alone. The Peyer’s patches and spleens of inoculated mice were examined on Days 4 and 10 after inoculation. In several experiments it was found that only χ4550 containing the intermediate copy number mZP3 construct pYA3229 could be recovered on Day 4 (10⁴ CFU), whereas no vaccine strain was detected in the spleen. Six days later, the vaccine strain had reached the spleen and Peyer’s patches with 1.5 × 10⁴ CFU and 2 × 10⁴ CFU, respectively. However, χ4550 with the pUC18-based mZP3 clone, pYA3202, was not recovered from inoculated mice. Western blot analysis of the colonies recovered from the mice inoculated with χ4550(pYA3229) demonstrated expression of recombinant mZP3 in all samples tested (data not shown).

Immune Responses in Mice Immunized Orally with S. typhimurium Expressing mZP3

Anti-ZP IgG antibodies were deposited on ZP in vivo as shown by direct immunofluorescence (Fig. 3). Antibody levels were determined by ELISA using solubilized whole ZP. No significant antibody titer to ZP protein was detected in the serum samples and vaginal washings from the first two collections, 5 and 7 wk after the initial immunization. Specific circulating antibodies of the IgG isotype were detectable in χ4550(pYA3229)-immunized mice after two booster immunizations (p < 0.01), with the highest titer at 4 wk after the last booster immunization (Fig. 4A). Antigen-specific antibody levels of the IgA isotype were also detected in the vaginal washings of χ4550(pYA3229)-immunized mice, with a peak titer at 2 wk after the last boost-

FIG. 3. IgG-type antibodies bound to the ovarian ZP detected by direct immunofluorescence in mice receiving a single oral inoculation of χ4550(pYA3229). A) Arrows point to antibody deposited on ZP. Zona-bound IgG antibody was not detected in mice immunized with χ4550(pYA3149) (B). Asterisk in B indicates unstained ZP under overexposed condition. ×500.

FIG. 4. Time course of production of antibodies to native ZP, measured by ELISA, in mice after three oral inoculations with either χ4550(pYA3229) (solid circles), χ4550(pYA3149) (open circles), or PBS only (squares). A) The titers of serum IgG antibody expressed as optical density at 490 nm; B) the titers of IgA antibody in vaginal washings. Bars indicate standard error.
er immunization, which gradually decreased over time (Fig. 4B). Data from each individual animal showed that 100% of immunized mice produced detectable IgG and IgA anti-ZP antibodies. In contrast, no antibodies to ZP3 were demonstrated in either of the control groups (Fig. 4). Pathological examination of the ovaries from immunized mice showed grade 0 on the disease index, indicating that no ovary inflammation was observed (data not shown).

**Fertility Trials in Female Mice Immunized with S. typhimurium Vaccine Strain Expressing mZP3**

As summarized in Table 1, all six female mice that received one immunization with χ4550(pYA3229) became pregnant with an average litter size of 7.67, which was similar to those of χ4550(pYA3149) and BSG control groups (7.67 and 7.50 pups per litter, respectively). One month after all the pregnant females had given birth (i.e., 15 wk after the first immunization), the mice were given two booster immunizations 1 wk apart. Nonimmunized male C3He mice were introduced 6 wk after the last boost and were kept with the females for four ovulation cycles.

Of six females immunized with χ4550 (pYA3229), three (50%) were not pregnant at the end of the trial (Table 1). The other three females gave birth to litters of normal size, thus giving an average litter size for the entire group of 4.33 pups. In contrast, all females (100%) in both the vector and BSG control groups became pregnant, delivering litters with an average of 8.5 and 8.2 pups per mouse, respectively. There was a significant difference (p < 0.05) between number of pups per litter (4.33 ± 4.93) in the ZP3 immunization group and number of pups per litter in the vector immunization group (8.17 ± 1.17). Moreover, the difference between pregnancy rate (50%) in ZP3-immunized mice and pregnancy rate (100%) in control mice was significant (p < 0.05). Contraceptive effects are represented by cumulative litter size in Figure 5, with 26 in the experimental group, 51 in the nonimmunization control group, and 49 in the vector control group by the end of the second trial.

During the two mating experiments, specific antibodies to ZP were monitored by collection of serum samples from the female mice prior to cohabitation. Results similar to those from the experiments on antibody response were obtained. There were no detectable antibody titers to ZP antigen for the first trial in which mice had been given a single immunization. Significant anti-ZP IgG antibodies (1:100 dilution, mean optical density value 0.098 ± 0.099, p < 0.01) were found in mice after they were given two booster immunizations in the second trial (Table 1). Correlation between antibody titer and litter size in individual mice was not observed at this time point.

After the fertility trial, ovaries were taken from mice immunized with mZP3 and tested for oophoritis. No evidence of ovarian disease was found by pathological examination; all mice tested were negative.

**DISCUSSION**

Even though various contraceptive methods are available today, rapid population growth argues for the need for user-friendly, cost-effective means of birth control all over the world. Immuncontraception, which remains an achievable but still challenging field of study, would be an efficient method of contraception [1, 2]. ZP is an extracellular structure that surrounds developing and mature oocytes. ZP3, one of the major glycoproteins in mouse ZP, plays a crucial role in fertilization as primary sperm receptor [6, 7]. It has been found that the presence of anti-ZP antibodies correlates with infertility in women [25], and mAb against ZP3 have been shown to block fertilization [7]. Therefore, ZP has been explored as a candidate immunogen in the development of a contraceptive vaccine [4, 5, 14]. Recombinant S. typhimurium, which is able to deliver heterologous antigens to the mucosal immune system to induce secretory IgA antibody responses, is a potential vector for a variety of oral vaccines [17], including those expressing gamete-specific antigens needed for fertilization. An oral contraceptive vaccine would have several advantages over conventional vaccines. Such a vaccine would be easier to administer, would induce mucosal antibodies in the reproduc-

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<th>Immunization</th>
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<th>Pregnancy rate (%)</th>
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* The mean optical density value from six mice for IgG antibodies was 0.098 ± 0.099 at 1:100 dilution (Student's t-test, p < 0.01).
tive tract, and after several immunizations would be effective over a long period of time. It may therefore be a more efficient method of contraception than current practical methods. Recently it was reported that oral immunization of mice with an avirulent *Salmonella*, which expressed a human sperm-specific antigen SP-10, induced both systemic and mucosal antibody responses in the reproductive tract [18]. The potential of an antifertility effect in that study, however, could not be evaluated, as the antibody response was directed against a human antigen. Using a similar strategy, we have cloned and expressed cDNA encoding murine ZP3 in an attenuated vaccine strain of *Salmonella*. This recombinant *S. typhimurium* induced both a serum and a mucosal antibody response to autologous ZP3 in immunized female BALB/c mice. More importantly, we were able to test the contraceptive effect in the immunized mice. Indeed, three oral immunizations with this vaccine strain led to infertility in three of six mice.

One of our strategies in the construction of an mZP3 vaccine was to optimize in vitro expression of mZP3 in *Salmonella*. We anticipated difficulties in the expression of nonfusion recombinant ZP3, as ZP3 is naturally insoluble and highly glycosylated. Indeed, the expression level of full-length mZP3 in *Salmonella*, though detectable by Western blot, was extremely low. Furthermore, in addition to the band of expected molecular size of ZP3 polypeptide by SDS-PAGE, there were three lower molecular size bands that were also recognized by the ZP3-specific mAb. No further characterization was done on these bands, since a potential protease cleavage site exists in mZP3 around the 352 amino acid residue [26] and this band may have been a degradation product of the recombinant ZP3 or interrupted translation. In order to increase expression level, the murine ZP3 cDNA sequence was then modified to delete not only the N-terminal 22 amino acid signal sequence but also the C-terminal 60 amino acid hydrophobic portion. Deletions at both ends of mZP3 led to an increase in the expression level of recombinant mZP3.

Since the expressed recombinant mZP3 was in a truncated form and without any glycosylation, its immunogenicity, especially for B cell epitopes, may be altered. A number of findings, however, support the view that antibodies to recombinant ZP protein might still exhibit contraceptive activity. For instance, it has been shown that antibodies directed against the polypeptide backbone of zona protein can interfere with sperm binding and penetration of ZP in vivo [27]. Immunization with fully or partially deglycosylated mZP3 results in reduced fertility in some species [13]. Some linear B cell epitopes of ZP3 have been mapped, and synthetic peptides coding those regions elicited antibody to native ZP and inhibited fertilization in vivo [14, 15]. In the present study, the recombinant mZP3 expressed by *Salmonella* was recognized by mAb IE-10, suggesting that some, if not all, important B cell epitopes such as ZP3 aa 327–342, had been preserved. Indeed, mice orally immunized with the recombinant *Salmonella* produced both IgG and IgA antibodies that recognized native ZP3, as demonstrated by ELISA using native ZP3 as coating antigen. More importantly, it was observed that elicited IgG antibodies were deposited on ZP in vivo. The affinity of antibodies induced by recombinant ZP3 for the native ZP3 and the specificity of these antibodies will need to be analyzed.

IgG antibodies to ZP3 in the circulation may be of major importance in antifertility, since it has been shown that IgG antibodies can penetrate into ovarian follicles and bind to ZP in vivo [27]. Although immunization with the ZP3-expressing *Salmonella* strain elicited an antibody response to native ZP3, the level of serum IgG antibody was much lower than that elicited by active immunization with the B cell epitope of ZP3 [14, 15]. Both the immunogenic nature of recombinant ZP3 and its expression level may be involved. In this regard, an *S. typhimurium* vaccine strain expressing SP-10 on a higher copy number Asd· plasmid vector elicited higher levels of circulating anti-SP10 IgG than were elicited with a lower copy number plasmid [18]. Although the pUC18-based plasmid with higher copy number replicon did direct higher levels of expression of ZP3 in vitro, that *Salmonella* vaccine strain failed to colonize the host's internal lymphoid organs in detectable numbers. We therefore had to switch to a pBR-based vector with lower copy number for vaccine construction.

Recently, several linear B cell epitopes of ZP3 have been mapped [28, 29]. They might be linked together and expressed as a multiple B-epitope polypeptide, which could be further fused with a bacterial protein to increase immunogenicity. It would also be possible to use a B-cell epitope to reduce the pathogenic T-cell response, thus reducing the possibility of ovarian pathogenesis. We have generated a fusion protein of one of these mZP3 epitopes, aa 327–342, with the B subunit of the heat-labile toxin (LT-B) of *E. coli* (unpublished results). Compared to controls, mice immunized with the *S. typhimurium* strain χ4072 (an asd· derivative of χ4064 [16]) expressing this fusion protein had a significant reduction in cumulative litter size after a booster immunization. These mice also had significant anti-LTB titers in both vaginal washings and serum.

When attenuated *Salmonella* was used to deliver a recombinant sperm-specific antigen SP-10, which is an allogenic antigen to female mice, it was predictable that they would mount an antibody response to the recombinant sperm proteins [18]. In contrast, ZP3 is a female self-antigen, and immune tolerance to ZP3 should be well established. Although heterogenic ZP protein(s) elicited antibody cross-reactive with self-ZP [9–11], immunization with autologous ZP protein in complete Freund's adjuvant in female rabbits failed to induce an antibody response [30]. Our study revealed that oral immunization of mice with *S. typhimurium* expressing recombinant mouse ZP3 induced both IgG and IgA autoantibodies that bound to endogenous ZP. This result implies that immune tolerance to this "self" antigen might be broken down by *Salmonella* immunization. Two possible mechanisms may explain the phenomenon. First, immunization with *Salmonella* may have a strong adjuvant effect that provokes ZP3-specific T cells to react with this self-antigen. Since endogenous ZP antigen does not induce B-cell tolerance [23], the B cells are ready to be activated upon the activation of ZP3-reactive T cells. Second, because the molecular structure of recombinant ZP3 is different in many ways from that of native ZP3, antigen processing of recombinant ZP3 may be different. It is well known that alteration of the processing of an antigen by antigen-presenting cells may result in the appearance of novel T-cell epitopes (cryptic T-cell epitope), to which no immune tolerance is established (see also see [31]). Thus, tolerance to self-protein is broken down.

Breakdown of self-tolerance to ZP3 is necessary to elicit anti-ZP3 autoantibodies, a major effector in immunocontraception. Simultaneously, it may also lead to activation of ZP3-specific T cells that in turn could cause autoimmune ovarian disease. It has been demonstrated that T cells per se (probably Th1 phenotype), not autoantibody, initiate the disease in a mouse model [27, 32]. For a contraceptive vac-
cine using a conventional immunization strategy, it will be extremely important to avoid inclusion of the harmful T-cell epitope with the useful ZP3 B-cell epitope [15]. Unlike the B-cell epitope, the T-cell epitope is restricted by major histocompatibility complex class II haplotype. It is almost impossible to predict the pathogenic T-cell response to autologous ZP3 in an outbred population. In the present study, although all of the females that were orally immunized with the recombinant Salmonella vaccine strain produced anti-ZP3 antibody, none of them had developed ovarian pathology at autopsy. This may not be unusual, as the BALB/c mice used in the present study are a nonresponder to an oophorogenic peptide of mouse ZP3 [30–34] [15]. It will therefore be necessary to investigate the disease induction in other strains of mice in the future. Because ovarian autoimmune disease induced by immunization of male mice with ZP3 peptide encoding a T-cell peptide may be reversible so that it may not be observable, we may have missed the correct timing to observe the disease. Since Th1 but not Th2 T cells relate to pathogenesis in organ-specific autoimmune disease models such as autoimmune ovarian disease [27, 31], experimental allergic encephalomyelitis [34], and NOD (non-obese diabetic) mice for diabetes [35], it is possible that the Salmonella strain used in the present study may direct a dominant Th2 response. Although some strains of Salmonella induce a Th1-type T-cell response [36], several investigations have demonstrated that a mitogen expressed by Salmonella may preferentially induce a Th2-type T-cell response [37]. It will be interesting to investigate whether or not the Salmonella strain used in this study induces a specific Th2-type immune response.

The results of the fertility trial with mice immunized with the recombinant Salmonella are encouraging. Three of six immunized females became infertile after three immunizations. In contrast, all mice that received only one immunization had no detectable antibody and became pregnant after one to two ovarian cycles, with litters of normal size. These results suggest that antibodies against ZP3 may play a role in infertility in the immunized mice. However, a correlation between antibody to ZP and litter size was not found in individual mice in this study. It is possible that the correlation might have been found in the mice from which serum samples were collected immediately after the copulation plugs were observed. Nevertheless, infertility in the mice immunized with recombinant Salmonella may not be due to pathology, as no ovarian inflammation was observed at autopsy before the mating experiment started or soon after the end of the fertility trial.

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REFERENCES

SALMONELLA VACCINE INDUCES ANTI-ZP Ab AND INFERTILITY


