The mechanism responsible for the loss of ovarian function is population, a situation from which the ovary cannot recover. This deficit arises from several causes, but it is likely that one critical event is the destruction of the primordial follicle population. The destruction of the primordial follicle population may be advantageous in the development of immunocontraceptive vaccines for wild-life population management. In contrast, depletion of the primordial follicle population may be disadvantageous in the development of immunocontraceptive vaccines for human use based on zona pellucida antigens. In contrast, depletion of the primordial follicle population may be advantageous in the development of immunocontraceptive vaccines for wild-life population management.

It has been suggested that the loss of ovarian function is due to an auto-immune mechanism mediated through ZP3-specific auto-reactive T cells. This has been extensively studied in a mouse model using defined peptides from mouse ZP3 (mZP3). Miller et al. (1989) identified a seven amino acid peptide mZP3316-342 with the potential to suppress fertility. High responding mice immunized with a mZP3328-342 peptide vaccine incorporating this epitope were infertile in the absence of any ovarian pathology. However, in certain strains of mice the same peptide induced auto-immune disease associated with a leukocytic infiltration into the ovary. A bone marrow-derived (B)-cell epitope (mZP3335-342) (Lou et al., 1995) and two nested thymus-derived (T)-cell epitopes that are thought to be responsible for the observed oophoritis, have now been identified within this region of the molecule (Garza and Tung, 1995). It has been demonstrated by Lou et al. (1995) that a chimeric peptide vaccine which incorporates this B-cell epitope (in a slightly modified form), with a foreign, promiscuous T helper cell epitope successfully induced reversible infertility in mice in the virtual absence of ovarian pathology. In view of these results we have undertaken to examine the

Design and evaluation of a ZP3 peptide vaccine in a homologous primate model

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The concept of a safe, immunocontraceptive vaccine using the zona pellucida glycoprotein 3 (ZP3) as an immunogen has been marred by the appearance of ovarian dysfunction in several species. However, careful selection of epitopes on mouse ZP3 have demonstrated that it is possible to segregate contraceptive bone marrow-derived (B)-cell epitopes from the cytotoxic thymus-derived (T)-cell epitopes thought to be responsible for inducing ovarian disease. B-cell epitopes on marmoset ZP3 (mstZP3) were identified by epitope mapping studies. Using a panel of polyclonal antibodies against recombinant mstZP3, an immunodominant epitope mstZP3301-320 was identified. A chimeric peptide was co-linearly synthesized incorporating this sequence with a promiscuous tetanus toxoid T-helper cell epitope. Using the common marmoset (Callithrix jacchus) as an animal model, we have compared the consequences of active immunization with homologous recombinant mstZP3 and mstZP3301-320 chimeric peptide vaccine. Long-term infertility was achieved using mstZP3 but at the expense of ovarian function. In contrast, no disruption to ovarian function was observed following mstZP3301-320 immunization. Antibodies to this peptide immunolocalized to the zona pellucida of both marmoset and human ovarian sections and inhibited human sperm–zona binding by ~60% in vitro. However, in-vivo studies indicated that targeting a single ZP3 epitope was insufficient to reliably and consistently achieve a contraceptive effect.

Key words: contraception/epitope mapping/immunization/zona pellucida/ZP3

Introduction

The unique specificity that surrounds sperm–egg interaction has been exploited in the development of immunocontraceptive vaccines that target the process of fertilization. The focus of many of these investigations has been the zona pellucida (ZP), an acellular glycoprotein shell that surrounds the oocyte and plays key roles in ensuring both fertilization and early embryonic development are successful (Kerr et al., 1998). It has been known for some time that antibodies raised against ZP can successfully inhibit sperm–egg recognition and binding in vitro, although the mechanisms by which these antibodies exert their contraceptive effect in vivo remains uncertain. Several studies have demonstrated that infertility can be achieved following active immunization with either native or recombinant ZP antigens (Sacco et al., 1987; Mahi-Brown et al., 1988; Paterson et al., 1992, 1998). However there is an accumulating body of evidence to suggest that antibodies directed against the major glycoprotein ZP3 cause infertility not only by an antibody-mediated disruption of sperm–egg interaction but also via a deleterious effect on ovarian function itself (Mahi-Brown et al., 1988; Paterson et al., 1992, 1998; Van de Voort et al., 1995; Jackson et al., 1998). This loss of ovarian function is characterized by a disruption of folliculogenesis and ultimately depletion of the primordial follicle population, a situation from which the ovary cannot recover. The mechanism responsible for the loss of ovarian function is currently under intense investigation and represents a key issue in the viability of any contraceptive vaccine for human use based on zona pellucida antigens. In contrast, depletion of the primordial follicle population may be advantageous in the development of immunocontraceptive vaccines for wild-life population management.
consequences of immunising the marmoset monkey (*Callithrix jacchus*) with homologous, glycosylated, recombinant ZP3. Antibodies thus generated were used to epitope map mstZP3 to identify homologous, immunodominant B-cell epitopes that would be available in the native structure. A continuous epitope was successfully identified in this study and incorporated into a chimeric peptide vaccine to evaluate its contraceptive efficacy and safety in a primate model.

**Materials and methods**

**Production of recombinant marmoset ZP3**

The cloning and sequencing of marmoset ZP3 into the cloning vector pCR1000 (Invitrogen, Leek, The Netherlands) has previously been described by Thillai-Koothan et al. (1993). Marmoset ZP3 cDNA was excised from the pCR1000 construct by EcoRI and KpnI digestion, purified and re-ligated into EcoRI and KpnI linearized Bluescript SK + vector (Strategene, Cambridge, UK) yielding pBluescript+MstZP3. From this construct the cDNA was digested with KpnI and BamHI and ligated into the expression vector pNKS1 which we have previously used to express human ZP3 in Chinese hamster ovary (CHO) cells (van Duin et al., 1994). The integrity of the construct (pNKS1.MstZP3) was checked by resistance enzyme analysis.

To transfect marmoset cDNA into CHO cells the calcium phosphate precipitation method of Graham and van de Ed (1973) was used. Six dishes containing $5 \times 10^6$ cells were treated with 10 µg pNKS1.MstZP3 cDNA/dish and the transfectants selected with medium containing 0.8 µg/ml Geneticin (Gibco, Paisley, UK). After 17 days, ~500 colonies/dish were observed and these were selected to be cloning by limiting dilution to give 1 cell/well. After 13 days the clones were grown for a further 2 days in serum-free culture medium and the supernatants tested for the presence of mstZP3 by dot blot analysis using rabbit anti-human ZP3 antisera. Two small, single colonies that gave an intense signal on dot blots, were selected and grown in roller bottles for the large scale production of mstZP3. Serum-free medium was concentrated to $4 \times 10^4$ times with Centricron-30 tubes (Amicon, Stonehouse, UK) and subjected to Western blot analysis using polyclonal antibodies raised against human ZP and mouse monoclonal antibodies (mAb) raised against human ZP (mAb 7A). These studies indicated that both recombinant marmoset and human ZP3 migrate at a molecular weight of 50–60 kDa and that the epitope recognized by mAb 7A is conserved between human and marmoset ZP3.

Marmoset ZP3 was therefore affinity purified using Protein G Sepharose 4B Beads coupled to mab7A as described previously (van Duin et al., 1994).

**Epitope mapping**

12-mer peptides, offset by four amino acids on each successive peptide and covering the entire sequence of marmoset ZP3 (Thillai-Koothan et al., 1993) were purchased from Chiron Mimotopes (Clayton, Australia); 108 peptides in total. The soluble peptides were synthesized in the following format ‘biotin-SGSG-peptide-NH$_2$’ to facilitate attachment to streptavidin-coated 96-well plates. The plates were prepared by dispensing a 100 µl aliquot of streptavidin at 5 µg/ml in distilled H$_2$O into each well of a 96-well plate and incubated at 37°C for 16 h. The plates were then blocked by adding 200 µl/well 0.01 M sodium phosphate, 0.15 M NaCl, 0.1% (v/v) Tween 20, pH 7.2 (PBS-T) for 1 h at 25°C. The plates were then washed by flooding the plate with PBS-T and vigorously flicking the solution from the wells. This procedure was repeated four times. The peptides (0.8 µmol) were dissolved in 400 µl of an appropriate solvent according to the sequence of the peptide. Just before use, a 1/1000 dilution of each peptide in PBS-T was prepared and 100 µl transferred to the appropriate well of a streptavidin-coated plate and incubated for 1 h at 25°C with shaking. The plates were then washed 4× with PBS-T as described above. A 100 µl aliquot of the serum to be tested was added to each well of the microtitre plate and incubated for 1 h on a shaker platform at 25°C. After washing four times with PBS-T, a 1/1000 dilution of rabbit anti-monkey immunoglobulin (IgG) peroxidase conjugate (Sigma, Poole, UK) in 1% rabbit serum, 0.1% Tween 20, 0.1% w/v sodium caseinate, 0.01 M phosphate-buffered saline (PBS) pH 7.2 was prepared and 100 µl added to each well. After incubating for 1 h at 25°C the plates were washed as before and a final wash of PBS only was applied. A 100 µl/well aliquot of substrate was added [0.05% ABTS (2,2’ azinobis (3-ethylbenzothiazolo-line-6-sulphonic acid) in 0.1 M citrate phosphate buffer, pH 4.0], incubated for 45 min and then measured at 405 nm.

The serum tested in this procedure was marmoset polyclonal antiserum raised against recombinant marmoset ZP3 used at 1/1000 dilution in PBS-T. In addition, four different marmoset monoclonal antibodies raised against recombinant human ZP3 and used at 1/250 dilution were tested and were a gift from Dr M. van Duin (NV Organon, Oss, The Netherlands; n = 4).

**Active immunization**

Prior to immunization all marmosets were monitored for at least 200 days to determine plasma progesterone concentrations. Only marmosets which displayed regular ovarian cycles were used in these studies. Twice weekly blood samples were taken from the femoral vein to determine both plasma progesterone concentrations and following primary immunization, antibody titres. Whilst antibody titres were being established, the animals were housed with a vasectomized male until mating studies began when the male was removed and replaced with a fertile male. Vaginal lavages were carried out to ensure mating was occurring regularly during the trial period.

**Recombinant marmoset ZP3 (mst ZP3)**

A primary injection of 25 µg mstZP3 and 250 µg N-acetyl-muramyl-l-alanyl-1-isoglutamine (MDP; Calbiochem-Novabiochem, Nottingham, UK) was emulsified in Morris adjuvant (Guilford Lab Ltd, Guildford, UK). The vaccine (total volume was 0.2 ml) was administered s.c. over three or four injection sites. Booster injections of 25 µg mstZP3 in Morris adjuvant were administered every 2 months. One month following the third booster injection mating studies commenced. Details of the immunization regime are outlined in Figure 1 (n = 6).

**hZP3**

Peptides representing the N-terminal sequence of hZP3 and mstZP3, following cleavage of the putative signal sequence, were synthesized with an additional cystine at the C-terminus. This domain was identified by epitope mapping human ZP3 and probing with a monoclonal antibody against human ZP known to suppress human sperm–egg interaction. The peptides hZP3$^{33-30}$ and mstZP3$^{33-30}$ were conjugated via the additional cystine residue to tetanus toxoid using the bi-functional reagent sulphosuccinimidyl 4-[(N-maleimidomethyl) cyclohexane-1-carboxylate]. Details of this conjugation method have been reported previously (Paterson et al., 1998). A primary injection of 200 µg peptide-TT conjugate and 250 µg MDP, emulsified in Morris adjuvant was administered s.c. After 2 months, a booster injection of 200 µg peptide-TT in Morris adjuvant was given and subsequent booster injections were given at 2 monthly intervals (n = 4 for each peptide).
A chimeric peptide was synthesized comprising a T-helper cell epitope from tetanus toxoid, a β-turn sequence and the mstZP3 301–320 sequence. The immunogen was co-linearly synthesized such that the T cell epitope was located at the amino terminus of the B cell epitope (with C-terminal amidation), separated by a four amino acid spacer (GPSL) to induce a β turn in the molecule. A primary injection of 100 µg peptide and 250 µg MDP, emulsified in Morris adjuvant was administered s.c. After 2 months, a booster injection of 100 µg peptide in Morris adjuvant was given and subsequent booster injections were given at intervals of 2 months. One month after the third booster injection, mating studies were initiated. Details of the immunization regime are outlined in Figure 2 (n = 4).

ELISA assays
Following the primary injection, blood samples were taken every 2 weeks to monitor antibody titres using an enzyme-linked immunosorbent (ELISA) assay. To determine antibody responses to mstZP3, 96-well microtitre plates (Nunc) were coated with 25 ng/100 µl/well mstZP3 in 0.1 M NaHCO3/Na2CO3, pH 9.6. After adsorption overnight at 25°C, the coating antigen was removed and the plates were washed for 3×5 min with PBS containing 3% bovine serum albumin (BSA) (PBS-A). To prevent non-specific binding, the plates were incubated with 200 µl PBS-A/well for 1 h at 25°C. Serum samples (50 µl/well) diluted in PBS, 0.1% BSA, 0.1% Tween-20 (TBS-T), were added in doubling dilutions from 1/10 to 1/1×107 and incubated at 25°C for 2 h. The wells were aspirated and washed three times with PBS-T. A 1/100 dilution of rabbit anti-monkey IgG horseradish peroxidase conjugate (anti-monkey HRP; Sigma) in PBS-T was added (50 µl/well) and incubated for 1 h at 25°C. After aspirating and washing as before, 50 µl of substrate solution (0.05% ABTS (2,2'-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid)) in 0.1 M citrate phosphate buffer, pH 4.0) was added and allowed to develop at 25°C for 15 min. The absorbance at 405 nm was determined by use of a microtitre plate reader. The titre end-point was taken to be the dilution that no longer gave an absorbance value greater than twice that of the negative control. The negative control was pooled serum taken from the same marmoset prior to immunization.

To assess anti-peptide titres, peptides corresponding to the sequence to be assayed were covalently linked to maleic anhydride microtitre plates (Pierce and Warriner, Chester, UK) through the peptide’s α-amino group at a concentration of 500 ng/100 µl/well in 40 mM phosphate buffer, pH 8.0. After incubating overnight at 25°C, the plates were aspirated, washed and the ELISA performed as described for mstZP3.

Competition assay
To assess the integrity of the anti-mstZP3 301–320 antibodies, a competition assay was performed. MstZP3 301–320 maleic anhydride microtitre
plates were prepared and blocked with PBS-A as described above. To each row a 50μl aliquot of mstZP3\(^{301-320}\) in PBS-T was added in doubling dilutions from 1 mM to 0.5 μM, then a 50μl aliquot of anti-mstZP3\(^{301-320}\) antiserum at 1/500 dilution in PBS-T was added to each well in the row. After incubating for 2h at 25°C the plates were aspirated and washed three times in PBS-T. The second antibody and substrates steps were then performed as described for mstZP3.

**Immunocytochemistry**

For immunocytochemical studies, marmoset and human ovaries (obtained, with consent, from patients undergoing hysterectomy) were fixed in 4% buffered paraformaldehyde, dehydrated with alcohol and subsequently embedded in paraffin. Sections of 5 μm were floated onto glass slides, rehydrated and rinsed in distilled water. These sections were incubated for 20 min with 3% hydrogen peroxide to quench any endogenous peroxidase activity. After washing for 20 min in 0.05 M Tris, 0.15 M NaCl, pH 7 (TBS) the ovarian sections were blocked by overlaying with 10% normal rabbit serum for 20 min in a humid chamber. A 1/10 dilution of marmoset antiserum was then added and incubated for 1.5 h at 25°C. The sections were washed for 3×5 min in TBS before incubation with a 1/250 dilution of anti-monkey HRP for 1.5 h at 25°C. The sections were then washed as before and overlaid with AEC substrate (3-amino-9-carbazole; Zymed, San Francisco, CA, USA) for 30 min, counter-stained with haematoxylin and mounted using ‘Aquamount’ (Merck Ltd, Lutterworth, UK). To confirm the specificity of antibody binding, antisera against the mstZP3\(^{301-320}\) chimeric peptide was pre-absorbed with 1 mM mstZP3\(^{301-320}\) peptide for 1 h before applying to the ovarian sections.

The sections were photographed using an Olympus AX70 microscope, Kodak DCS 420 digital camera and Adobe Photoshop software (Adobe Systems Inc, Mountain View, CA, USA).

**Human sperm–zona binding assay**

Human oocytes that had failed to fertilize during in-vitro fertilization (IVF) treatments were kindly donated for research purposes with the patients’ consent. The ova were stored at 4°C in 1.5 M magnesium chloride, 0.1% (w/v) dextran, 20 mM HEPES buffer until required.

Human spermatozoa were obtained from semen samples donated by a panel of healthy, normozoospermic donors (World Health Organization, 1992) after 2 or 3 days abstinence. The samples were...
produced into sterile containers and left for at least 30 min to liquefy before being processed by discontinuous Percoll gradient centrifugation through 50 and 100% Percoll (Aitken et al., 1995). The 100% fraction was resuspended to a final concentration of 1×10^7/ml in HEPES-buffered Biggers–Whitten–Whittingham medium (BWW) (Biggers et al., 1971) supplemented with 0.3% Albumin (Armour Pharmaceutical Company, Eastbourne, UK). The spermatozoa were capacitated by incubating in 3 mM pentoxifylline in BWW for 3 h, prior to incubation with the oocytes.

For the assay, the oocytes were washed several times in BWW medium to remove the high salt storage medium and placed in a 100 µl droplet of BWW medium under liquid paraffin. The antisera to be tested (all with equivalent titres) were diluted 1/5 with BWW. Incubation dishes were prepared by placing 100 µl of test antiserum in a 60 mm plastic Petri dish along with three 100 µl droplets of BWW under liquid paraffin at 37°C. The oocytes were transferred to the antiserum droplet and incubated for 3 h at 37°C in the presence of 5% CO₂. The oocytes were then washed by transferring through the three 100 µl droplets of BWW, pipetting each oocyte several times through a fine bore glass pipette to remove unbound antiserum. A second set of incubation dishes was prepared by placing 100 µl of sperm suspension (5×10^6/ml) in a 60 mm plastic Petri dish along with three 100 µl droplets of BWW under liquid paraffin. The oocytes were transferred to the sperm droplet and incubated for 1.5 h at 37°C in the presence of 5% CO₂. The oocytes were then washed as before by transferring through three 100 µl aliquots of BWW and pipetting each oocyte several times through a fine bore glass pipette to remove any loosely adhering spermatozoa.

To visualize the bound spermatozoa a 1 mg/ml solution of Hoechst 33258 fluorochrome (Sigma) in dimethyl sulphoxide was prepared and protected from light. A working solution of 1 µg/ml H33258 in 0.025% glutaraldehyde in 20 mM Tris, pH 8.0 was used to fix and stain the spermatozoa. The oocytes were transferred to 50 µl aliquot of stain and washed through 100 µl aliquots of 20 mM Tris, pH 8.0. The bound spermatozoa were visualized and counted under UV light using a Leitz DMRB microscope.

A minimum of six ova were used in each experiment and each experiment was replicated six times giving a minimum of 36 oocytes for each treatment.

**Human ZP immunocytochemistry**

Salt-stored human oocytes were washed free of their storage medium and re-suspended in a 100 µl droplet of BWW medium containing a 1/25 dilution of the antiserum to be tested under paraffin oil. After 3 h at 37°C the oocytes were washed three times in BWW and transferred to a 100 µl aliquot of a 1/1000 dilution anti-monkey-HRP in BWW for 1 h at 37°C. The oocytes were washed three times then placed in 100 µl AEC substrate for 15 min, washed a further three times then finally mounted on slides and photographed as described for ovarian immunocytochemistry.

**Western blot analysis**

Salt-stored human oocytes were washed free of their storage medium and re-suspended in 0.0625 M Tris, pH 6.8. The oocytes were incubated at 78°C for 10 min to dissolve the zonae pellucidae, then centrifuged at 8000 g for 20 min and the supernatant retained for analysis. Recombinant human ZP3 (10 µg) marmoset ZP3 (10 µg) and heat-solubilized human ZP (10 oocytes/lane) were added to an equal volume of sample buffer (0.0625 M Tris, pH 6.8; 2% (v/v) SDS; 10% (v/v) glycerol; 2% (v/v) mercaptoethanol and heated at 100°C for 5 min. The samples were loaded onto 10% SDS polyacrylamide gels and electrophoresed at 30 mA constant current. The protein were transferred onto nitro-cellulose (Towbin et al., 1979) at 25 V constant voltage for 16 h. Western blot analysis was subsequently performed using polyclonal marmoset antiserum at 1/500 dilution, anti-mouse HRP as second antibody (1/6000) and an enhanced chemiluminescence detection technique (Amersham, Little Chalfont, UK). To confirm the specificity of anti-mstZP3, this antiserum was pre-absorbed with mstZP3 pre-absorbed with mstZP3 (0.5mM) for 1 h before applying to the nitrocellulose.

**Results**

**Production of marmoset ZP3**

Marmoset ZP3 was successfully expressed by CHO cells. Silver stained SDS–PAGE gels demonstrated that high purity material was obtained by affinity purification using a monoclonal antibody raised against human ZP (Figure 3a). No differences could be detected between recombinant human and marmoset ZP3 on silver stained gels, and Western blotting analysis under reducing conditions indicated that both molecules migrated with a molecular weight of 50–60 kDa (Figure 3a,b). In addition to the predominant 50–60 kDa band several low molecular weight components were recognized by antiserum against mstZP3 and, to a lesser extent, mstZP3 pre-absorbed with mstZP3 peptide (0.5mM) for 1 h before applying to the nitrocellulose.
Active immunization with hZP323–30 and mstZP323–30

High anti-peptide responses were observed in all marmosets and no disruption of ovarian function was observed. Anti-hZP323–30 antisera cross-reacted with the corresponding human peptide and recombinant hZP3 but did not cross-react with recombinant mstZP3 (Figure 4a). In contrast, anti-mstZP323–30 recognized the corresponding marmoset peptide but no cross-reactivity was observed with either recombinant human or marmoset ZP3 (Figure 4b). In addition anti-mstZP323–30 antibodies recognized hZP323–30 peptide and vice versa (data not shown). This indicates that a change in a single, critical, amino acid is sufficient, possibly by inducing a conformational change in the molecule, to prevent antibodies against a marmoset peptide recognizing human ZP3. Consequently all further studies were carried out using the homologous primate model.

Active immunization with mstZP3

Recombinant, glycosylated, marmoset ZP3 expressed in CHO cells proved to be a very effective immunogen as all marmosets responded to the immunization protocol. High anti-mstZP3 antibody titres of 1/100 000 were achieved in five out of six animals (Figure 1a–e) and these marmosets were infertile for the duration of the study. However in each of these marmosets the resulting infertility was associated with the onset of ovarian pathology as determined by a gradual cessation in cycling plasma progesterone concentrations, that ultimately fell to baseline values. This loss of ovarian activity was only observed after long-term active immunization. However, it appeared to be related to antibody titres as ovarian pathology was observed as early as 200 days post-immunization in two marmosets whose antibody titres were rapidly elevated above 1/100 000 (Figure 1d,e). Ovarian sections from these marmosets indicated a severe bi-lateral pathology, in particular a complete cessation in folliculogenesis was apparent and a virtual depletion of the primordial follicle population. Disruption in plasma progesterone concentrations was not observed in the sixth animal (Figure 1f) which had the lowest antibody titres. This marmoset conceived ~150 days after mating studies began.

Marmoset ovarian sections probed with anti-mstZP3 antibodies immunolocalized to the outer surface of marmoset ZP3 placing them in an ideal location to disrupt sperm–egg recognition and binding (Figure 5a). In addition small islands of ZP3 immunoreactive material were observed on primordial follicles. This observation has implications for the future reversibility of any ZP3 based vaccine but was not observed with the anti-mstZP3301–320 antisera.

Epitope mapping studies

The results of epitope mapping marmoset ZP3 with marmoset antisera (n = 4) raised against recombinant marmoset ZP3 are shown in Figure 6a–d. A number of powerful, continuous, immunodominant domains within mstZP3 sequence were apparent. Although some domains were unique to an individual marmoset’s serum, there were several epitopes that were common to all anti-mstZP3 antisera. No cross-reactivity was observed with these epitopes when pre-immune marmoset sera was tested (n = 2; Figure 6e). One immunodominant region in particular, representing three 12-mer peptides covering the sequence mstZP3301–320, was recognized not only by all four polyclonal antisera tested but also by a marmoset monoclonal antibody raised against recombinant human ZP3 (M.Paterson, Z.A.Jennings, M.R.Wilson et al., unpublished). In addition, immunocytochemical studies demonstrated that this monoclonal antibody recognized and bound exclusively to native ZP on marmoset ovarian sections. This sequence therefore, was taken further for evaluation of its contraceptive potential in vivo.

Active immunization with mstZP3301–320 peptide

Four marmosets were immunized with a chimeric peptide immunogen consisting of the mstZP3301–320 peptide and a promiscuous T-helper cell epitope from tetanus toxoid (residues 582–599) (Lairmore et al., 1995). Structural analysis of the chimeric peptide indicated that the amino acids within the T cell epitope assumed an amphipathic α-helix, which is thought to be required for T helper cell function; the B cell epitope

![Figure 4. Representative antibody titre profile following active immunization with (a) hZP323–30 TT (n = 4) and (b) mstZP323–30 TT (n = 4). P = primary immunization; B = booster immunization. Antisera was tested by enzyme-linked immunosorbent assay (ELISA) against the following antigens: (a) hZP323–30; hZP3; mstZP3; and (b) mstZP323–30; hZP3; mstZP3.](https://academic.oup.com/molehr/article-abstract/5/4/342/1141045/10.1093/oup/mehr/hte198)
had an irregular or loop conformation. This combination of an amphipathic α-helix and a loop region is thought to be important for chimeric peptide vaccine design (Eroshkin et al., 1993).

This was an effective immunogen as all four marmosets responded to a primary immunization of the mstZP3301–320 chimeric peptide and produced antibody titres of 1/10 000 following subsequent booster injections (Figure 2). The integrity of anti-mstZP3301–320 antibodies was demonstrated by a competitive ELISA in which binding of these antibodies was inhibited by mstZP3301–320 peptide in a dose-dependent manner (Figure 7). The ability of anti-mstZP3301–320 antisera to recognize the mstZP3301–320 peptide and the entire mstZP3 protein was compared and in each antisera tested (n = 4) it was demonstrated that the observed anti-peptide titre was higher than that of the entire mstZP3 molecule (Figure 8). Analysis of ovarian function by monitoring plasma progesterone concentrations indicated no disruption of normal cyclicity following long-term active immunization with this chimeric mstZP3 peptide.

Significantly this particular peptide was successful in generating antibodies that cross-reacted with the marmoset zona pellucida. On marmoset ovarian sections these antisera immunolocalized particularly to the outer surface of the zona pellucida (Figure 5b) and on human ovarian sections a uniform staining throughout the zona pellucida was observed (Figure 5c). This is the first demonstration of an antiserum to a marmoset ZP3 peptide that cross-reacts with both human and marmoset ZP3. The ability of antisera against mstZP3301–320 to recognize the native zona pellucida of isolated salt-stored human oocytes was confirmed by immunocytochemistry (Figure 9).

In addition, the ability of antibodies against the mstZP3301–320 peptide to cross-react with primate ZP3 was confirmed by Western blotting techniques employing both

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**Figure 5.** Immunocytochemistry of marmoset (a, b, d, e) and human (c, f) ovarian sections. These sections were probed with marmoset anti-sera (a) anti-mstZP3 antisera showing intense immunolocalization to the outer surface of marmoset ZP; (b) anti-mstZP3301–320, again showing enhanced immunolocalization to the outer surface of marmoset ZP however, no staining of primordial follicles was observed; (c) anti-mstZP3301–320 showing uniform staining throughout the zona pellucida of a human ovarian section; no staining was observed with pre-immune marmoset sera on either (d) marmoset or (f) human ovarian sections or (e) following pre-absorption of anti-mstZP3301–320 antisera with mstZP3301–320 peptide. Scale bar = 50 µm.
Figure 6. Epitope mapping of marmoset ZP3. 12-mer peptides were sequentially synthesized, offset by four amino acids on each adjacent peptide. The peptides were biotinylated at the N-terminus to facilitate attachment to streptavidin-coated enzyme-linked immunosorbent assay (ELISA) plates and then probed with anti-mstZP3 raised in four individual marmosets (a–d). The hatched bars represent three peptides covering the immunodominant epitope mstZP3301–320 that was selected for further evaluation; (e) pre-immune serum.

Figure 7. Enzyme-linked immunosorbent assay (ELISA) assay showing the competitive inhibition of binding of anti-mstZP3301–320 antibodies at 1/100 dilution when co-incubated with increasing concentrations of mstZP3301–320 peptide: --- anti-mstZP3301–320 titre; □ = anti-mstZP3 titre.

Figure 8. Comparison of anti-mstZP3 and anti-mstZP3301–320 titres using pooled antisera from four marmosets (a–d) immunized with mstZP3301–320. ■ = anti-mstZP3301–320 titre; □ = anti-mstZP3 titre.

recombinant human and marmoset ZP3 and heat solubilized human zona pellucidae (Figure 3b).

Zona binding studies
The presence of antibodies against mstZP3301–320 bound to the outer surface of human zonae pellucidae significantly inhibited binding of human spermatozoa to the surface of the zona pellucida by ~60% when compared with control pre-immune serum (P < 0.005; Mann–Whitney) as shown in Figure 10. Interestingly, a slightly greater inhibition of sperm binding was observed with antibodies to the immunodominant peptide than with antibodies to the entire glycosylated marmoset ZP3 molecule. It is possible that the restricted repertoire of antibodies raised against the mstZP3301–320 peptide is directed against a functional domain in terms of sperm–egg interaction. However this observation is not statistically significant when compared across all four anti-mstZP3301–320 antisera tested. When antibodies to recombinant human ZP3 were used in the homologous sperm–egg binding assay as a positive control, an almost complete inhibition of sperm binding was observed. Although a significant suppression of sperm binding was observed in vitro this translated to a slight reduction in the fertility of these marmosets in vivo. While one marmoset remained infertile for the duration of the experiment, the remaining three marmosets conceived within two cycles of mating studies commencing. The normal conception rate for this marmoset facility is 96% (K.Morris, personal communication).

Discussion
The high sequence identity between human and marmoset ZP3 (91% identity at the amino acid level, Thillai-Koothan et al., 1993) has made the marmoset monkey (C.jacchus) the animal of choice in our in-vivo studies in the development of immunoc contraceptive vaccines based on ZP3 glycoproteins and ZP3 peptides (Paterson et al., 1992, 1998). However, active immunization studies using the human and marmoset peptide from ZP3301–320 have indicated that a single change in a critical amino
Acid can have a dramatic impact on epitope recognition, possibly due to a conformation change in the molecule. We have demonstrated that a single amino acid change (W→R: h→mst) completely prevented antibodies against a marmoset peptide from binding to human ZP3 according to ELISA (Figure 4) and vice versa. Furthermore, in our previous studies employing human ZP3 peptides (Paterson et al., 1998), anti-peptide antibodies were induced that cross-reacted with recombinant human ZP3 on ELISA but exhibited little cross-reactivity with the native ZP structure on marmoset ovarian sections.

As a consequence of these studies we have examined the contraceptive potential of ZP3 using a completely homologous model involving the immunization of marmosets with recombinant, glycosylated mstZP3. Antisera from these animals have then been used, in conjunction with marmoset monoclonal antibodies, to epitope map mstZP3 to identify homologous, continuous, immunodominant, B-cell epitopes. From these analyses the domain mstZP3301–320 was chosen to evaluate the potential of this peptide vaccine to induce infertility in the absence of any ovarian pathology.

Active immunization with recombinant glycosylated mstZP3 produced high antibody titres, despite being a self antigen, which resulted in infertility in five out of six marmosets (Figure 1). However this contraceptive effect was achieved at the expense of an ovarian pathology characterized by the cessation of folliculogenesis and depletion of the primordial follicle population. This association between infertility and either a transient or irreversible loss of ovarian function has been demonstrated in several non-human primates (Dunbar et al., 1989; Sacco et al., 1991; Paterson et al., 1992, 1998). More recently, infertility was achieved in mice following immunization with a Salmonella typhimurium vaccine strain expressing mouse ZP3 (Zhang et al., 1997) and a live ectromelia virus expressing ZP3 (Jackson et al., 1998). In the latter case, infertility was associated with disruption in ovarian follicular development in ~50% of mice. In agreement with our own findings, this ovarian pathology was not associated with a lymphocytic infiltration into the ovary. This loss of ovarian function may be due to several reasons. Firstly, the depletion of primordial follicles may be the result of antibodies binding to developing follicles and disrupting folliculogenesis via an antibody-dependent, cell-mediated cytotoxicity or complement-mediated lysis. As described here and in other studies (Grootenhuis et al., 1996; Paterson et al., 1998) small islands of ZP3 immunoreactive material have been observed on primordial follicles and this may make them particularly susceptible to immunological attack. Antibodies binding to the surface of the zona pellucida could also interfere with the close communication between oocytes and the surrounding granulosa cells via gap junctions. Cell to cell signalling through these inter-cellular channels is thought to be critical in regulating the highly co-ordinated set of events which are required for follicular development (Simon et al., 1997).

Several studies using a mouse model have suggested a possible mechanism for ovarian autoimmune disease following the induction of immunity against ZP3 which centres on autoreactive T-cell epitopes within this molecule. Rhim et al. (1992) were able to demonstrate that oophoritis could be induced in naive mice by transfer of CD4+ T-cell lines from affected animals immunized with mZP3328–342 without any demonstrable antibody response to the native ZP structure. Two nested T-cell epitopes have now been identified within...
mZP3^{330–342} (Garza and Tung, 1995). The segregation of the B- and T-cell response in this region of mouse ZP3 has enabled Lou et al. (1995) to induce reversible infertility in mice in the virtual absence of oophoritis. This was achieved by immunizing mice with mZP3^{335–342} in which an amino acid, critical for a T-cell response, was modified and incorporating a foreign promiscuous T-helper cell epitope.

We have epitope mapped marmoset ZP3 to identify immunodominant B-cell epitopes for evaluation of their contraceptive efficacy in the homologous primate model (Figure 6). The region mstZP3^{301–320} was selected for further investigation as it satisfied several criteria: (i) it is a continuous, immunodominant epitope; (ii) this domain was recognized by anti-mstZP3 serum from all animals tested; (iii) a marmoset monoclonal antibody which mapped to this region recognized native ZP on immunocytochemistry. A chimeric peptide was chosen for the vaccine in which a promiscuous helper T cell epitope from tetanus toxoid was co-linearly synthesized, N-terminal to the ZP3 peptide. This vaccine design was chosen as we have previously demonstrated that tetanus toxoid is an effective carrier protein (Paterson et al., 1998) and it has been reported that the location of a T cell epitope at the N-terminus of the B-cell epitope induces a higher affinity antibody than the reverse orientation (Partidos et al., 1992).

This chimeric vaccine was a very effective immunogen as high antibody titres against mstZP3^{301–320} were attained in all marmosets (Figure 2). In this region of ZP3 the human and marmoset sequences are identical apart from a single amino acid change at position 305. The ability of these anti-peptide antibodies to bind to recombinant human and marmoset ZP3 as well as the native ZP structure were clearly demonstrated by Western blotting, immunocytochemistry and zona binding studies. This is the first demonstration of anti-serum to a marmoset ZP3 peptide that can cross-react with both native marmoset and human ZP and is encouraging for the development of immunocontraceptive vaccines for human use.

This particular region of ZP3 has also been identified in porcine ZP by Afzalpurkar et al. (1997). Following conjugation to diptheria toxoid the resulting antibodies were able to recognize pZP3β but did not inhibit porcine sperm–oocyte interaction. However, when this antisera was used in combination with antiserum against other epitopes on pZP3β, significant disruption of porcine sperm–egg interaction was observed. Afzalpurkar et al. (1998) have obtained similar results using bonnet monkey ZP3^{300–322}, antiserum against this region bound to the ZP of bonnet monkey ovarian sections but failed to block human sperm–egg interaction. When antiserum was raised against a cocktail of bonnet monkey ZP3 epitopes, including ZP3^{300–322}, a positive co-operative interaction was observed resulting in a significant reduction in human sperm–egg binding. Kaul et al. (1997) also induced antibodies to bonnet-monkey ZP3^{300–322} and found that the resulting antisera bound to the homologous recombinant ZP3.

We conclude from these studies that the polypeptide backbone of primate ZP3 contains powerful, immunodominant B cell epitopes with the potential to generate high titre antibodies targeting the zona pellucida. Furthermore, the presence of antibodies directed against ZP3 peptides in vivo is fully compatible with normal ovarian function, particularly when a comparison of anti-peptide antisera showed that the titre of the anti-peptide response was greater than the response against the entire mstZP3 molecule. This result emphasizes that ovarian pathology is not invariably associated with high antibody titres against the zona pellucida. Although a reduction in human sperm–egg binding of >60% was observed in vitro, mating studies indicated only a slight suppression in fertility. The generation of antibodies targeting a single ZP epitope therefore appeared to be insufficient to occlude sperm binding in vivo and hence achieve a consistent contraceptive effect. This strategy however provides the basis for future multi-epitope ZP3 vaccines with the potential to disrupt the earliest stages of fertilization in the absence of ovarian pathology.

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