Review of Literature
The Zona Pellucida-Ultrastructure and Development

Mammalian oocytes are covered by a thick, acellular, translucent glycoprotein matrix known as the ZP which mediates diverse and important functions during early mammalian development. The ZP determines the primary species specific interaction of sperm with the oocyte, while the oolemma is generally more permissive to interspecies fertilization (Yanagimachi, 1977). Sperm-ZP interaction involves specific events of recognition in which one or more sperm membrane receptors interact with their ligands on the ZP. Following fusion of the sperm with the oocyte, a rapid electrical depolarization of the egg membrane (Sato, 1979) followed by the cortical reaction inactivates the sperm receptor thereby blocking polyspermy. Moreover, ZP also protects cleaving embryos as they traverse the female reproductive tract prior to implantation. The ZP is comprised of three glycoproteins which are biochemically and immunologically distinct and are classified as ZP1, ZP2 and ZP3, and also on the basis of mRNA size as ZPA (longest), ZPB and ZPC (shortest) (Harris et al., 1994a).

The ZP surrounding fully grown mouse oocytes is ~7 μm thick and contains 3-4 ng of protein (Bliel and Wassarman, 1980a). Under the electron microscope, the ZP shows a lattice network with fenestrations revealing an inner compact layer with small pores and an outer loosely arranged layer with larger pores. Specific MAbs based localization and chemical cross linking experiments have shown that the mouse ZP (mZP) matrix comprises 2-3 μm long interconnected filaments each containing a 14-15 nm structural repeat which is thought to be a ZP2-ZP3 heterodimer with a ZP1 homodimer cross-linking the filaments (Greve and Wassarman, 1985). The porcine ZP (pZP) is about 16 μm thick and contains 30-35 ng of protein (Dunbar, 1983).

Development of the ZP layer begins with the deposition of homogenous extracellular material between the growing oocyte and adjacent granulosa cells (Zamboni, 1976; Dvork and Tesarik, 1980). The deposits then coalesce to form confluent layers around the oocyte. However, microvilli from the oocyte and processes from the granulosa cells remain embedded in this matrix. The communication and interdigitation of these
processes increases as the oocyte and granulosa cells differentiate, increasing the surface area of contact between the two cells and forming channels through the ZP providing nutrition to the developing oocyte.

**ZP Glycoproteins-Biochemical Characterization**

The ZP proteins have been analyzed for size after separation by gel electrophoresis or column chromatography. Under non-reducing condition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of murine ZP reveals three families of glycoproteins, ZP1 (180-200 kDa), ZP2 (120-140 kDa) and ZP3 (83 kDa) (Bleil and Wassarman, 1980a). The hZP shows ZP1 (90-110 kDa), ZP2 (64-76 kDa) and ZP3 (57-73 kDa) (Shabanowitz and O'Rand, 1988). The pZP under non-reducing conditions shows ZP1 (80-90 kDa) and ZP3 (55 kDa) and under reducing condition resolves into ZP1 (82 kDa), ZP2 (65 kDa), ZP3 (55 kDa) and ZP4 (21 kDa) (Yurewicz et al., 1987). Porcine ZP1 (pZP1) revealed immunological cross-reactivity with ZP2 and ZP4, which do not share reactive epitopes amongst themselves, thereby suggesting that they are derived from reduction of disulfide bonds in ZP1. pZP3 family consists of a mixture of two biochemically and immunologically distinct glycoproteins, pZP3α (core protein 37 kDa) and pZP3β (core protein 32 kDa). Two dimensional gel electrophoresis shows that each family exists as several isoelectric species. The pZP3 family consists of 20 charge isomers with apparent PI values of 3.5-6.0. This charge heterogeneity has been attributed to differential glycosylation and not to the polypeptide backbone (Hedrick and Wardrip, 1981).

The carbohydrate content of ZP glycoproteins is 30-40%. The N- and O-linked oligosaccharides (OS) are of branched type, rich in sulfated polylactosamines. Purified pZP3α and pZP3β have been obtained following partial enzymatic deglycosylation by endo-β-galactosidase (Yurewicz et al., 1987) or by complete chemical deglycosylation (Henderson et al., 1987a). Much attention has been directed in recent years to the OS components of ZP glycoproteins since several lines of evidence suggest that the sperm bind to the OS of the ZP and not to the polypeptide backbone per se (Macek and Shur, 1980b).
1988; Wassarman, 1992; Litscher and Wassarman, 1993). Both O-linked (Florman and Wassarman, 1984; Yurewicz et al., 1991) and N-linked OS (Nakano et al., 1996) have been implicated in this interaction by different investigators.

**Gene Structure**

The genes for ZP3 glycoproteins have been cloned and sequenced from several species. On the basis of mRNA size the ZP glycoprotein family has been classified as ZPA (longest), ZPB and ZPC (shortest) (Harris et al., 1994a).

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<tr>
<th>ZPA family</th>
<th>Human ZP2</th>
<th>(Liang and Dean, 1993)</th>
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<tr>
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<td>(Harris et al., 1994b)</td>
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<td>Bonnet monkey ZP2</td>
<td>(Jethanandani et al., 1997)</td>
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<td>Dog ZPA</td>
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<td>Mouse ZP2</td>
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<td>(Harris et al., 1994a, b)</td>
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<tr>
<td>Marmoset ZP3</td>
<td>(Thillai-Koothan et al., 1993)</td>
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<tr>
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<td>(Ringuette et al., 1988; Kinloch et al., 1988)</td>
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Mouse ZP1 (mZP1), ZP2 (mZP2) and ZP3 (mZP3) are single copy genes located on chromosomes 19, 7 and 5 respectively (Lunsford et al., 1990; Epifano et al., 1995b). A comparison of the ZP genes between mouse and human reveals interesting features.
mZP2 spans 12.1 kb having 18 exons ranging in size from 45-190 bp and 17 introns (81-1490 bp), and is transcribed into a 2201 nt mRNA with short 5' (30 nt) and 3' (32 nt) untranslated regions coding for a polypeptide of 713 aa residues (Liang et al., 1990). Human ZP2 (hZP2) gene is composed of 19 exons (one more than the mouse) and the transcript contains an ORF of 2235 nt encoding a polypeptide of 745 aa residues with a nt sequence identity of 70% with mZP2 (Liang and Dean, 1993). The predicted hydropathicity and α-helical content of the two proteins are similar (Liang et al., 1990). mZP1 has a single open reading frame (ORF) of 1869 nucleotides (nt) encoding a 623 aa polypeptide as compared to the 540 aa polypeptide of human ZP1 (hZP1, ZPB). hZP1 as compared to mZP1 is least conserved (43% aa sequence identity) due to an elongated exon 3 in the mouse gene. mZP3 gene is 8.6 kbp and has 8 exons (92-338 bp) and seven introns (125-2320 bp) (Kinloch et al., 1988; Chamberlin and Dean, 1989). It is transcribed into a 1317 nt ZP3 mRNA and also has short 5' (29 nt) and 3' (16 nt) untranslated regions. Human homologue of the mouse sperm receptor spans 18.3 kb containing 8 exons, sizes of which are conserved between the two species and both contain a single ORF coding for a 424 aa polypeptides which share an identity of 67% (Chamberlin and Dean, 1990).

Taken together these data suggest that the overall structure of the zona genes and their gene products are conserved among mammals. The three zona proteins share common structural motifs. Each has an N-terminal signal peptide which directs these into a secretory pathway and is cleaved off from the mature polypeptide chain. Each of the zona proteins also has a transmembrane-like domain near its C-terminus about 34-47 aa downstream of a potential tetra basic furin cleavage site, R-X-R-R. Proteolytic processing of this hydrophobic transmembrane-like domain may play a role in intracellular trafficking of these secreted proteins or their interactions in the extra cellular matrix.

There are 20 conserved cysteine (Cys) residues, and 1 or 2 non conserved ones in the ZP2 (ZPA) sequences studied. The non conserved Cys residues occur either at the N-
terminal leader region or C-terminal region where a large amount of variation among the ZP2 sequences occurs. The ZP1 sequences seem to be more divergent than the ZP2 or ZP3 (ZPC) groups. There are 21 conserved Cys residues in the ZP1 sequences. There are regions of homology between the ZP1 and ZP2 class proteins indicating the involvement of a common ancestral gene (Harris et al., 1994a). Comparison of the aa sequences of the ZP3 genes reveals three regions of disparity; i) the putative leader sequence (20-25 aa of the mature protein); ii) the region containing the epitope (aa 336-342) recognized by a MAb against mZP3 that blocks fertilization (Millar et al., 1989); and iii) the C-terminal region of the protein after the furin processing site. There are 14 conserved Cys residues besides one or two extra ones in the ZP3 family. The extra Cys residues are near the N- or the C-terminus which are the regions of maximum heterogeneity in the protein. The conservation of the Cys residues indicates an overall conserved backbone structure of the ZP proteins.

Despite considerable sequence identity and very similar size of the polypeptide core, differential glycosylation leads to considerable heterogeneity in the molecular mass of the secreted ZP proteins. Mouse and hamster ZP3 (hamZP3) sharing a sequence homology of ~82% have very different apparent molecular weights of ~83 and ~56 kDa respectively (Moller et al., 1990). The regions of maximal heterogeneity in the ZP3 polypeptide chain and differences in post-translational glycosylation might contribute to the species specificity of sperm-oocyte interaction.

Investigators using mammalian systems have assumed the ZP glycoproteins to be unique to mammalian species. Evidence that the glycoproteins are conserved not only through mammalian evolution but through the entire vertebrate evolution has been accumulating. Cloning and sequencing of the vitelline envelope (VE) glycoproteins, which surrounds the plasma membrane in fish oocytes has revealed considerable homology with the mammalian ZP proteins. The primary structure of medaka (Oryzias latipes) egg VE polypeptide called L-SF41 was found to be significantly similar to the ZP3 polypeptide (Murata et al., 1995). Moreover, 10 of the invariant Cys residues have
been conserved in this species indicating the overall conservation of the three-dimensional structure of the ZP family through evolution. In flounder (*Pseudopleuronectes americanus*) the gene encoding another VE protein called wffemale shares considerable sequence homology with the ZP2 protein family. Analysis of the sequence has revealed that exons 11-16 in the mouse and exons 2-7 in the fish wffemale gene have been derived from a common ancestor (Liang et al., 1990; Lyons et al., 1993). The fish are the oldest of the vertebrates and the observed similarities between the ZP and VE polypeptides bears directly on the evolution of the ZP proteins. However, in teleost fish, unlike the ZP glycoproteins, precursors of the VE are synthesized in the liver and transported to the growing oocytes.

In the anuran, *Xenopus laevis*, homologues of the ZP glycoproteins ZP1, ZP2 and ZP3 have also been identified sharing extensive homology with human, porcine and mouse sequences (Hedrick, 1996). Comparison of the translated aa sequences of the Xenopus ZP2 gene with the human, porcine and mouse revealed homologies of 28.5%, 27.6% and 26.9% respectively. The ZP1 and ZP3 genes were relatively better conserved with homologies of 41.6%, 41.7% and 36.8% with human, porcine and mouse homologous ZP1 sequences and 40.9%, 40.0% and 40.8% with the human, porcine and mouse homologous ZP3 sequences respectively.

**Regulation of Expression**

Regulation of expression of ZP genes has been extensively studied in the mouse model system where expression is restricted to the oocytes (Ringuette et al., 1986, Liang et al., 1990; Epifano et al., 1995a, b). Additionally, synthesis of ZP is oocyte specific as shown by 3H-fucose and 35S-methionine labeling experiments (Blief and Wassarman, 1980b). Data also indicates that there is ordered expression of the zona glycoproteins during oogenesis (Epifano et al., 1995b). ZP2 transcripts can be detected in oocytes before birth, well before the growth phase of oogenesis, as early as day 16 of gestation (Millar et al., 1993). ZP1 and ZP3 transcripts, however, are detected only after the oocyte enters the 2 week growth phase prior to ovulation. As oocytes grow, all three zona transcripts
accumulate and in the 50-60 μm oocyte represent ≈1.5% of the total poly(A)+ RNA. ZP1 is the least abundant of the transcripts and represents 25% of the ZP2 or ZP3 which are present in roughly equimolar amounts. Post ovulation, oocytes have less than 5% of the peak amounts of the zona transcripts. The concurrent accumulation of the ZP transcripts suggests that common transcriptional regulatory events may be involved in the regulation of gene expression.

Analysis of the upstream sequences of mouse and human ZP2 and ZP3 gene reveals the presence of 5 short conserved DNA sequences upstream of the TATAA box (I, IIA, IIIB, III, IV). Mutation analysis has revealed that the 12 bp element IV is both necessary and sufficient for high level expression of a reporter gene in mouse oocytes. Oligonucleotides corresponding to the conserved upstream regulatory elements from either ZP2/ZP3 form DNA-protein complexes of identical mobility in gel retardation assays (Millar et al., 1991) indicating the involvement of similar regulatory processes in expression. A putative transcription factor, zona pellucida gene activating protein-1 (ZAP-1) binding to the conserved element IV has been implicated in the positive regulation of ZP expression. The onset of mZP2 transcription as well as the profile of its subsequent accumulation correlates with the ZAP1 DNA binding activity (Millar et al., 1993). An oocyte specific 60 kDa protein (OSP-1), binding to nucleotides -99 to -86 of the mouse promoter, has also been identified and has been proposed as an oocyte specific transcription factor (Schickler et al., 1992). By effecting an inhibition of the de novo biosynthesis of zona proteins by antisense oligonucleotides, it was established that abolishment of either ZP2 or ZP3 protein synthesis, prevented the incorporation of the other protein into the extracellular zona matrix suggesting that ZP2 and ZP3 proteins are independent of each other in their biosynthesis but are dependent upon each other for incorporation into the zona matrix (Tong et al., 1995).

In situ hybridization of cynomolgous monkey ovaries with digoxigenin-labeled cDNA probes specific for the mRNA encoding for ZP1, ZP2 and ZP3 revealed the presence of mRNA encoding ZP2 in growing follicles at all stages and in the granulosa cells of
mature preovulatory follicles (Martinez et al., 1996). It has been reported that ZP1 is present in secondary and tertiary follicles and absent in primordial, primary and antral follicles as well as granulosa cells. ZP3 is present in oocytes at all stages of folliculogenesis. By using monoclonal and polyclonal Abs to r-hZP3, presence of ZP3 on primordial follicles and in granulosa cells of human ovaries has also been shown (Grootenhuis et al., 1996). This is suggestive of the production and secretion of hZP glycoproteins at specific stages of folliculogenesis and of a contribution from the granulosa cells to ZP2 and ZP3 synthesis. The contribution of ZP2 and ZP3 by granulosa cells in the non-human primates and humans is at variance with the observations in the mouse where these are specifically present only in the oocytes.

Expression of ZP Proteins

The difficulty in obtaining ZP glycoproteins from native sources in a pure form, sufficient for functional and immunogenicity studies, necessitated the use of heterologous expression systems. Expression of mZP3 in stably transfected L-292 and green monkey CV-1 cell lines under the cytomegalovirus or the vaccinia (P11) promoters resulted in r-proteins with a lower molecular weight (60-70 kDa) than native ZP3 (83 kDa). r-mZP3 was biologically active exhibiting both the sperm binding and the acrosome reaction inducing activities and was able to inhibit sperm-zona binding with a potency equivalent to that of the native protein (Beebe et al., 1992). However, when r-hamZP3 and r-mZP3 were expressed in mouse embryonic carcinoma cells these were glycosylated to very different extents (r-hamZP3=49 kDa; r-mZP3=83 kDa) such that they resembled their native counterparts. However, though r-mZP3 was biologically active, inhibiting the binding of sperm to ovulated eggs and inducing the acrosome reaction, r-hamZP3 was not biologically active (Kinloch et al., 1991). Transgenic mouse lines harboring the hamZP3 under the mZP3 upstream regulatory sequences and expressing hamZP3 at levels comparable with the endogenous mZP3 expression have been developed (Kinloch et al., 1992). The transgenic oocyte secreted and synthesized a glycoprotein indistinguishable from the native hamZP3 and incorporated both glycoproteins into a
mosaic ZP, hamZP3 purified from the mosaic oocytes exhibited both sperm receptor and acrosome inducing activities in vitro and following fertilization got inactivated (Kinloch et al., 1992). r-hZP3 (hZP3) expressed in CHO cells was expressed at a molecular weight slightly lower than the native protein and was capable of inducing the acrosome reaction and promoting fusion of human sperm to zona free hamster oocytes (van Duin et al., 1994). The rabbit 55 kDa and a fragment of the 75 kDa protein were expressed as cro-β-galactosidase fusion proteins in E. coli (Schwoebel et al., 1991; Lee et al., 1993). The rabbit 55 kDa protein has also been expressed in the baculovirus expression system (BV55). The r-BV55 protein is glycosylated and binds to rabbit sperm in the acrosomal region in a manner consistent with the sperm receptor activity. Anti-BV55 Abs blocked sperm-egg interaction, though the Fab fragment failed to do the same, suggesting that steric hindrance to the receptor site could be responsible (Prasad et al., 1995; 1996).

Expression of various ZP glycoproteins such as the pZP3β in bacteria, canine ZP3 in bacteria and BEYS and feline ZP1, ZP2 and ZP3 in bacteria has also been documented in an international patent document (Harris et al., 1994b).

**Sperm Receptor Function**

Although still speculative in nature, molecular mechanisms for sperm-egg adhesion and fusion during mammalian fertilization are emerging. ZP possesses a component that functions as a 'sperm-receptor' recognized by free swimming sperm in a species specific manner (Yanagimachi, 1994). The interaction between the spermatozoa and the oocyte initiates a complex cascade of events leading to fertilization and embryo formation.

Interaction is initiated by the recognition of complementary receptors on the gametes. The acrosome intact capacitated spermatozoa penetrate the layers of cumulus cells surrounding the ovulated egg. Contact with the ZP induces the spermatozoa to undergo the acrosome reaction (Yanagimachi, 1978), an exocytotic event induced by homologous ZP, characterized by the fusion of sperm plasma and outer acrosomal membrane, leading to the release of the acrosomal contents including a variety of hydrolyzing enzymes such as hyaluronidase, phosphatases, glycosidases, lipases and proteases. Progesterone may
also act in a synergistic fashion with ZP3 in the induction of the acrosome reaction (Roldan et al., 1994). Acrosome reaction also leads to a rearrangement of the spermatozoa surface topography permitting continued adhesion as well as penetration (Yanagimachi, 1994). The acrosome reacted spermatozoon penetrates the ZP and enters in the perivitelline space where it fuses with the plasma membrane of the oocyte (Yanagimachi, 1978). In mammals, penetration of the ZP by spermatozoa is possible only after they undergo the acrosome reaction (Anderson et al., 1975) while heterologous fertilization is possible only when the ZP has been denuded (Yanagimachi, 1984). The ZP thus acts as a checkpoint of the species specificity of fertilization.

Once a spermatozoon fertilizes an oocyte, subsequent binding of other sperm is inhibited as a result of the cortical reaction leading to the hardening of the ZP, imparting resistance to the action of proteases, low pH and reducing agents.

Purification of each ZP glycoprotein to homogeneity and evaluation of its function has revealed that ZP3 is the primary sperm receptor. In mouse purified ZP3 from ovulated eggs can induce the acrosome reaction in capacitated sperm (Bleil and Wassarman, 1986). Inclusion of relatively low concentrations of ZP3 and not ZP1 or ZP2, in an in vitro sperm-egg binding assay decreased the number of sperm bound to oocyte in a dose dependent manner. The observed inhibition is apparently caused by competition of the added ZP3 for complementary receptors on the mouse sperm head (Wassarman, 1992). However, ZP3 from fertilized eggs can not induce the acrosome reaction, suggesting modification of the sperm receptor following fertilization. Conversely, acrosome reacted sperm do not bind to ZP3. More recent studies using r-ZP3 proteins expressed in heterologous expression systems have further confirmed that in the mouse, hamster and human, ZP3 acts as the primary sperm receptor (Bleil and Wassarman, 1988; Moller et al., 1990; van Duin et al., 1994).

It has also been shown that mice which are homozygous for an insertional mutation in the ZP3 gene lack a ZP and are infertile (Rankin et al., 1996). The homozygous mutant ZP3−/− mice had follicles with germinal vesicle intact oocytes but lacked the ZP and had a
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disorganized corona radiata. On mating with males of proven fertility, none of the mutant mice produced offspring, implying a critical role for the ZP3 in the formation of the sperm-receptor site. Alternately, disruption of the mZP3 gene by targeted mutagenesis using homologous recombination in mouse embryonic stem cells resulted in production of oocytes lacking a ZP and accompanying infertility (Liu et al., 1996).

Several lines of evidence suggest that free swimming sperm recognize and bind to ZP3 OS and not to the polypeptide (Macek and Shur, 1988; Wassarman, 1992; Litscher and Wassarman, 1993). However, considerable controversy remains regarding the precise identity of the sugar residues involved in this interaction. Various lectins, a class of proteins that bind to carbohydrate moieties with high affinity and specificity, inhibit or abolish binding of sperm to zona-intact eggs (Oikawa et al., 1974). Classical experiments using enzymatic digestion of purified ZP3 and incubation of resulting fragments with capacitated sperm had revealed that small glycopeptides in the range of 1.5 to 6 kDa retained sperm receptor function (Florman et al., 1984). Deglycosylation of ZP3 by trifluoromethanesulfonic acid (removes both N- and O-linked OS from the polypeptide) led to loss of sperm receptor activity suggesting an important role for carbohydrates in the formation/function of the sperm receptor site. Modification of ZP3 by selective removal of N-linked OS by digestion with enzyme endoglycosidase-F and leaving the O-linked sugars in place, retained the sperm receptor activity implicating an important role for O-linked sugars in sperm binding function. Altering the molecule by removing the O-linked sugars by extensive digestion with pronase or alkaline reduction abrogated sperm receptor activity (Florman et al., 1984; Florman and Wassarman, 1984; Wassarman, 1989). For specific identification of the nature of the O-linked OS, ZP3 was subjected to treatment with different exoglycosidases. Preincubation of mouse sperm with \( \alpha \)-galactosidase and \( \alpha \)-fucosidase treated ZP3 failed to inhibit sperm-egg binding. Regeneration of the alcohol on C6 position by borohydride reduction on terminal galactose residue restored sperm receptor activity after it was lost following oxidation of C6 alcohol to an aldehyde by treatment with galactose oxidase (Bleil and Wassarman,
1988). Synthetic O-linked related OS constructs possessing Gal at their non-reducing terminus were effective inhibitors at fM concentrations of mouse gamete adhesion in vitro (Litscher et al., 1995).

Other studies, however, suggest that α-linked galactosyl residues may not be important for sperm adhesion to ZP. N-acetylglucosaminyl residues on mZP3 have been demonstrated to be recognized by galactosyl transferases present on mouse spermatozoa (Miller et al., 1992). Moreover, mice deficient in glycosyltransferase enzyme which is responsible for Galα1→3Gal synthesis and expression are fully fertile (Thall et al., 1995). A 55 kDa glycopeptide from the C-terminal portion of the mZP3 has been attributed with a sperm receptor function (Rosiere and Wassarman, 1992). In mice, a cluster of serine residues to which the sperm receptor O-linked chain(s) may be linked is localized in the C-terminal region encoded by exon 7. Glycosylation of one or more of the five serines clustered in the region, is required for ZP3 to bind sperm (Kinloch et al., 1995; Liu et al., 1995; Wassarman and Litscher, 1995).

In the porcine model, sperm receptor activity of chemically deglycosylated ZP3 (mixture of pZP3α and pZP3β) is lost (Berger et al., 1989) but retained in the endo-β-galactosidase treated portion (Sacco et al., 1989a). Further preincubation of spermatozoa with ZP3, endo-β-galactosidase digested ZP3 (EBGD ZP3) or ZP3α can inhibit sperm-egg interaction but preincubation with ZP3β and deglycosylated ZP3 (ZP3DG) does not (Sacco et al., 1989a), thus providing evidence for the importance of pZP3α. It has also been shown that O-linked OS from pZP3 can inhibit binding of boar sperm to porcine oocytes more efficiently than N-linked OS (Yurewicz et al., 1991). However, others have shown that sperm-egg binding in pigs is mainly mediated by tri- or tetra-antennary, neutral, complex-type N-linked carbohydrate chain(s) located in the N-terminal region of pZP3α protein (Nakano et al., 1996). Since the efficiency of this inhibition was seen to be far less than that caused by the whole molecule on a molar basis, it has been suggested that while carbohydrates might act as the substrate for the complementary molecules on the sperm, actual recognition might be a function of the
orientation of these carbohydrates on the protein backbone influencing the spatial contribution which is recognized as the sperm receptor. Recent observations, however, indicate that pZP3β, which is the homologue of hZP3, associates with pZP3α (homologue of hZP1) leading to high molecular weight hetero complexes which bind with very high affinity to boar sperm associated zona receptors thereby suggesting that both the glycoproteins participate in the sperm binding process (Yurewicz and Sacco, 1996).

A variety of putative zona receptor molecules on sperm have been characterized as probable receptors for sperm binding. Extensive studies in the mouse have resulted in the identification of several putative receptors. Amongst the antigens which have been proposed by investigators to serve as potential receptors on mouse spermatozoa are, sperm surface galactosyltransferase (Miller et al., 1992), fucosyltransferase (Ram et al., 1989), a trypsin inhibitor sensitive site (Benau and Storey, 1988), a ZP3-binding 56 kDa sperm protein (Cheng et al., 1994; Bookbinder et al., 1995), a 95 kDa sperm plasma membrane protein that has been suggested to be a unique hexokinase (Leyton and Saling, 1989) and a 115 kDa α-D mannosidase present on the plasma membrane of sperm from several species (Tulsiani et al., 1989; 1990). A trypsin like serine protease (acrosin/proacrosin) present in the sperm acrosome as well as on the surface of spermatozoa, has long been believed to have a receptor-like role in the mouse and pig (Yonezawa et al., 1995). However, it has been demonstrated that sperm from mice carrying a targeted mutation of the acrosin gene can bind to and penetrate mouse oocytes (Baba et al., 1994).

Human spermatozoa are known to posses an α-D mannosidase (Tulsiani et al., 1990) and a mannose binding protein (Benoff et al., 1993). It has been suggested that each of these macromolecules has a receptor like role in binding to mannose-containing OS on ZP. Fertility antigen-1 (FA-1) (Kadam et al., 1995) and a selectin like molecule (Dell et al., 1995) on human sperm has been shown to bind to homologous ZP.
Using immobilized pZP as an affinity medium, a species specific antigen, zonadhesin has been identified that is expressed by haploid spermatids and is homologous to both von Willebrandt factor and mucins. The purified protein has a molecular mass of 150 kDa with 2 non-identical subunits of 105 and 45 kDa (Hardy and Garbers, 1995). Receptor like roles have also been proposed for other porcine sperm membrane proteins including a fucose binding protein, a 53 kDa fucosylated protein that interacts with the carbohydrate moiety of ZP glycoproteins, sperm protein 38 and adhesin protein (APz) (reviewed by Tulsiani et al., 1997).

The continued binding and penetration has been attributed to the secondary sperm receptor, the ZP2, which binds preferentially to the acrosome reacted sperm. The ligand for ZP2 on the sperm has been suggested to be either PH20 as demonstrated in the guinea pig (Primakoff et al., 1985) or acrosin in pig (Yonezawa et al., 1995). ZP2 also acts as the substrate for the action of the proteolytic enzymes following the cortical reaction. It has been proposed that following fertilization, proteolysis of ZP2 results in the formation of small molecular weight fragments which do not dissociate but remain non-covalently bound (Wassarman, 1987). This increase in non-covalent interactions make ZP resistant to proteolytic cleavage and acts as a protective barrier. ZP1 has a role as a cross linker between ZP2 and ZP3, probably contributing to structural integrity of the ZP. However, the exact functional role of ZP1 in the process of fertilization is not yet understood. Recently, it has been shown at least in the mouse model, that the initial adhesion event between spermatozoa and the ZP is of a high affinity which involves interaction of multiple ligands. It has been reported that there may be as many as 30,000 binding sites with a density of 300 molecules/μm² (Thaler and Cardullo, 1996).

Once in the perivitelline space, sperm make contact with the oocyte and adhere. Adherence is followed by cell fusion and release of the oocyte from cell cycle arrest. There is now considerable evidence that this adhesion of mammalian sperm and oocyte is mediated atleast partially through the interaction of integrins on the oocyte and their cognate ligands, the disintegrins on sperm. The first sperm disintegrin, PH-30 was found
on the posterior head of guinea pig sperm. PH30 is a heterodimeric membrane protein with 2 homologous subunits, the α-subunit, containing a 22 aa region (residues 90-111) that resembles a viral fusion protein and the β subunit, possessing the disintegrin domain (Wolfsberg et al., 1995). Mammalian oocyte integrins are heterodimeric membrane proteins composed of unrelated α and β subunits. There are currently 14α and 8β integrins subunits known. There is agreement that those mammalian oocytes examined (mouse, hamster and human) all contain α6β1 integrins on their surface (Almeida et al., 1995; Fusi et al., 1993). In vitro fertilization can be inhibited by peptide analogues that serve as ligands for the integrins (Myles et al., 1994)

Binding of spermatozoa to ZP leads to an increase in the intracellular Ca++ (Florman, 1994). ZP3 induces the activation of a G-coupled adhesion molecule which may lead to the production of IP3 and subsequent activation of a voltage gated Ca++ channel in the spermatozoa plasma membrane (Snell and White, 1996). Recently, a change in the binding pattern of lectins, when stained with the complex Ricinus communis lectin-colloidal gold, has been reported following fertilization of rat oocytes. The activated eggs revealed uneven staining of the ZP of fertilized or activated eggs as compared to homogenous staining in the ovulated eggs (Raz et al., 1996).

Zona Pellucida: Potential as a Target Antigen

A number of criteria need to be fulfilled in order for any antigen to be considered as a candidate for contraceptive vaccine development viz., specificity to the reproductive process, effectiveness across a large population, accessibility to humoral and cell mediated immune systems, ease of availability in a large and sufficiently pure quality, minimal and acceptable side effects and most importantly, reversibility (Griffin, 1989). The ZP glycoproteins meet most of these criteria (Sacco, 1987) and by virtue of their critical role in the early events of fertilization, targeting the ZP glycoproteins for immunological neutralization of their function leads to a block in fertility. Ovaries unlike the testes are not immunologically sequestered organs and hence systemic Abs have easy access to the oocytes. Moreover, since only one egg is released during ovulation in each
cycle, Ab titres sufficient for the neutralization of only a limited amount of ZP antigen would be required. The antigens constituting the ZP envelope are not encountered in the blood, hence the danger of the formation of immune complexes is also negated. The ZP antigens are immunogenic and are also relatively well conserved across different animal species allowing for the development of animal models where the antigens may be tested in a homologous/heterologous system.

**ZP Antibodies**

Porter (1965) immunized female guinea pigs with guinea pig ovarian homogenates and demonstrated that the Abs generated reacted specifically with ovarian tissue. This study suggested for the first time that tolerance to ovarian self antigens could be overcome. Females did not conceive after artificial insemination and ovarian examination revealed ovulation failure. In another study, it was demonstrated that rabbit antisera against hamster ovarian homogenates could cause a surface precipitation of hamster oocytes treated *in vitro* and proteolytic digestion of ZP with pronase could be delayed (Ownby and Shivers, 1972). By adsorption of the sera with non-ovarian tissues, it was established that the primary ovarian antigen recognized by these Abs was the ZP. In another study, it was shown that oocytes treated with adsorbed antisera failed to bind sperm on the ZP (Shivers et al., 1972). Treatment of fertilized oocytes or early embryos with the antisera blocked implantation of the embryos (Dudkiewicz et al., 1975). Passive treatment of mice or hamsters with homologous Abs induced transient infertility (Jilek and Pavlov, 1975; Oikawa and Yanagimachi, 1975). Abs generated against crude pZP preparations showed cross reactivity with the ZP of mouse, rat, rabbit, dog, monkey and human (Sacco et al., 1981). It was also demonstrated that Abs against pZP could inhibit fertilization of human oocytes (Trounson et al., 1980).

**Active Immunization with ZP**

With methods being developed for the large scale purification of native zona antigens (Dunbar et al., 1980; Brauer and Sotelo, 1983; Yurewicz et al., 1987), immunization with relatively pure native proteins became possible and it became obvious that while
immunization with homologous ZP gave a low-titer response, immunization with heterologous ZP clearly proved to be highly immunogenic (Gwatkin et al., 1977; Wood et al., 1981; Mahi-Brown et al., 1982). pZP became the antigen of choice in early active immunization studies since it had the advantage of easy availability and immunological cross-reactivity with several species (Sacco, 1977; Yurewicz et al., 1983; Maresh and Dunbar, 1987; Mahi-Brown and Tung, 1995). Active immunization of female rabbits and bitches with cross-reactive ZP induced infertility (Wood et al., 1981; Mahi-Brown et al., 1982). In rabbits, superovulation by the administration of exogenous gonadotrophin did not restore ovulation and microscopic examination revealed the destruction of oocytes in all the growing follicles and severe depletion of the pool of resting follicles (Skinner et al., 1984). Granulosa and thecal cell clusters were also seen without oocytes. It was thus established that infertility was not a consequence of inhibition of sperm-egg interaction but rather due to an inhibition of follicular development. In another study, immunization of dogs with pZP was also accompanied by abnormal estrous cycles characterized by prolonged proestrus or estrus (Mahi-Brown et al., 1982). Furthermore, oocytes recovered from the ovaries of the immunized bitch failed to bind sperm. Histological examination of the ovaries of the immunized animals revealed, that as in the rabbit study mentioned above, animals with the highest titres showed depletion of oocytes.

Immunization of primates with crude preparations of pZP also rendered the animals infertile. The lack of reversibility, increased rates of follicular atresia as well as abnormal hormonal profiles were attributed to impure antigenic preparations, especially contamination with granulosa cell processes (Gulyas et al., 1983). Immunization of rhesus and bonnet macaques with porcine solubilized isolated ZP led to anovulatory endocrine profiles and wedge biopsies of ovaries revealed a complete absence of large antral or growing follicles (Suman et al., 1986). However, when the anti-ZP Ab titres declined, the animals regained normal ovulatory profiles clearly implicating ZP immunization as the cause for ovarian dysfunction. However, in another study,
immunization of marmoset monkeys with pZP did not lead to any ovarian pathology (Fox et al., 1981).

pZP has also been tested for immunocontraception in several wildlife species. In feral horse and donkey populations a single annual booster was capable of maintaining contraception, without affecting the complex social behavior of the feral species. Short term treatment was reversible while long term treatment (5-7 years) was associated with some ovulation failure and depressed urinary estrogen levels. Seventy four species of captive zoo animals have also been tested with the pZP vaccine with documented success in 27 of the 28 species for which data was available (Kirkpatrick et al., 1996).

Subsequently, studies were directed towards evaluating the effect of purified pZP3 as immunogen. This resulted in alleviation of adverse effects on ovarian function to a considerable extent. Purified pZP3 was used to immunize squirrel monkeys which generated a high titred Ab response and the animals remained infertile on mating with males of proven fertility (Sacco et al., 1987). This study revealed an initial disturbance in normal ovarian steroid secretion and function. Histological examination of the ovary suggested an interference with folliculogenesis. However, with time, ovarian function appeared to recover despite high anti-pZP3 Ab titres suggesting that although immunization with pZP3 initially produces a disturbance in normal ovarian function, these effects were reversible. Other than a slight depletion in the number of follicles there was no gross ovarian pathology and the steroid hormone profiles also remained normal. When female bonnet monkeys (Macaca radiata) were immunized with purified pZP3 or pZP3 conjugated to the β subunit of human chorionic gonadotrophin (hCG) along with adjuvants permissible for human use, it was observed that the animals generated good anti-pZP3 Ab titres, continued to have ovulatory cycles and remained infertile in the presence of high anti-pZP3 titres (Bagavant et al., 1994). There was also no disturbance in cyclicity save for summer amenorrhea. Laparoscopic examination revealed normal ovaries with developing follicles or corpora lutea on the surface. Following a decline in Ab titres, 50% of the animals became pregnant and in the other 50% that failed to regain
fertility, none of the ovaries showed any signs of inflammation or lymphocytic infiltration and there was also no increase in the number of atretic or degenerating follicles.

The effect of glycosylation on the pathology was also studied. In a comparative study performed using rabbits immunized with solubilized pZP3 as well as partially and highly deglycosylated pZP3α and pZP3β, ovarian histology revealed that only the pZP3β component induced ovarian pathology and it was also dependent on the presence of sugar chains on the immunogen while the pZP3α component did not cause changes in ovarian histology (Keenan et al., 1991; Jones et al., 1992). However, immunization with deglycosylated pZP3β in the marmoset monkey was associated with a decrease in the primordial follicular pool in the ovary (Paterson et al., 1992). Decreased estrogen concentrations followed by cessation of ovulation was observed in baboons immunized with deglycosylated pZP3 (Dunbar et al., 1989). Yet, the role of OS in inducing ovarian damage remains controversial. Studies in rabbits have shown that immunization with deglycosylated pZP causes ovarian pathology only if all the three proteins are present in the antigen preparation (Bhatnagar et al., 1992).

**Adjuvants**

ZP glycoproteins are only weakly immunogenic when injected without adjuvants (Bhatnagar et al., 1989). Earlier, studies largely employed Freund's complete adjuvant (CFA). However, the induction of granulomatous lesions at the point of immunization renders it unsuitable for human use (Warren et al., 1986). In bonnet monkeys, immunization with pZP3 along with CFA resulted in ovarian atrophy, whereas the ovarian morphology of animals immunized using aluminum hydroxide gel (alum) and SPLPS was normal (Upadhyaya et al., 1989). Adsorption of pZP antigens on alum along with CP20, 961, a synthetic lipid amine, as an emulsifier resulted in variable Ab titres. One of the animals developed a temporary inflammatory response to the adjuvant at the site of injection, developed the lowest Ab response, and conceived when bred, while 3 others produced higher Ab titres, remained infertile and did not show any inflammatory response (Mahi-Brown et al., 1985). Alum was, however, not effective in inducing an
adequate Ab response in squirrel monkeys (Sacco et al., 1989b). Use of synthetic muramyl dipeptide (MDP) derivative has been reported in many studies involving primates (Sacco et al., 1991; Dunbar et al., 1989, Paterson et al., 1992; Mahi-Brown et al., 1992; Bagavant et al., 1994). However, as compared to CFA, MDP does not induce high or sustained titres (Sacco et al., 1989b) though it is effective in inducing an immune response to ZP antigens capable of blocking fertility. On comparison of studies carried out with different adjuvants as in bitches immunized in alum or with CFA (Mahi-Brown et al., 1985) or squirrel monkeys immunized with alum, MDP or CFA (Sacco et al., 1989b), it was evident that CFA gave the best response. Studies carried out of late have reported the use of a synthetic block copolymer (TiterMax) as an adjuvant which does not lead to lesions at the site of injection as with CFA for immunization of cynomolgous monkeys (Mahi-Brown, 1996).

Monoclonal Antibodies to ZP

Earlier studies had shown that passive transfer of rat MAb s to murine ZP3 in female mice conferred infertility, thereby showing that targeting to a single epitope relevant to fertilization, could also bring about a contraceptive effect (East et al., 1984). Hence it was imperative to delineate the determinants involved in sperm-egg binding which would aid in the design of epitope based synthetic peptide vaccine immunogens. Towards this end, a panel of MAb s having specificity for pZP3α (12) and pZP3β (13) were generated (Bagavant et al., 1993; Chadha et al., 1993; Gupta et al., 1993; Gupta and Gupta, 1994). The in vitro contraceptive efficacy of these Ab was tested and it was determined that MAb-7, -28, -412 and -420 which are specific to pZP3α and MAb-30, -454, -455 and -467 having a specificity for pZP3β inhibited the attachment of boar sperm to porcine oocytes in vitro (Bagavant et al., 1993; Gupta et al., 1993; Gupta et al., 1995, 1996). It was shown that the inability of the other MAb s to inhibit sperm attachment to respective MAb-treated zona encased oocytes was not due to their non-reactivity with the ZP. Inhibition of sperm-egg binding affected by MAb s was neither a function of the affinity of the Ab nor its isotype (Bagavant et al., 1993). It was thus suggested that the epitopic
regions recognized by these Ab are either directly involved in sperm-egg interaction or are very close to the sperm receptor site. Some of these bioeffective Abs (defined as having an inhibitory effect on sperm-egg binding in vitro) viz., MAb -412 and -420 (specificity for pZP3α) and MAb -454, -455, -467 and -30 (specificity for pZP3β) recognized deglycosylated and reduced and carboxyamidomethylated form of respective antigens in Western blots, suggesting a recognition of linear epitopes on the protein backbone not stabilized by disulfide bonds. Epitope mapping strategies have revealed the binding domains of some of these Abs (Gupta et al., 1996; Afzalpurkar and Gupta, 1997). Recently, using a cocktail of immune sera generated against synthetic peptides corresponding to epitopes mapped by MAb on the pZP3β, adhesion of boar sperm to porcine oocytes could be inhibited in vitro (Afzalpurkar et al., 1997a). Moreover, antisera generated against the cocktail of analogous peptides based on the bZP3 sequence also inhibited the attachment and penetration of human spermatozoa to Ab treated oocytes in vitro (Afzalpurkar et al., 1997b). Evaluation of these regions as immunogens could yield useful information for the development of a safe zona contraceptive vaccine.

**Peptide Vaccines**

Gene cloning and epitope mapping studies have allowed the identification and use of synthetic peptide based vaccines. This approach allows a precise targeting of the humoral and the cellular arms of the immune response and hence may help circumvent the problem of T-cell mediated ovarian pathology. The disadvantage of this approach lies in the genetic restriction of the immune response. Moreover, in some cases the Abs generated may not react with the native protein. The efficacy of a ZP3 based synthetic peptide as an immunocontraceptive agent was first demonstrated in the mouse model (Millar et al., 1989). A rat MAb against murine ZP3 was identified which inhibited the in vitro sperm-egg interaction and could cause infertility in mice on passive transfer (East et al., 1985). Screening of peptide fragments of murine ZP3 with this MAb led to the identification of a 7 aa epitope (336-342) (Millar et al., 1989). Randomly bred female
NIH Swiss mice were immunized with a 16 mer synthetic peptide (328-343 aa residues) conjugated to keyhole limpet haemocyanin (KLH) encompassing the epitope of the rat MAb as well as an additional adjacent N-terminal 8 mer sequence. Abs were raised which recognized ovarian ZP and rendered 75% of the mice infertile for a period ranging from 1-9 months. Ovarian function remained normal and some animals regained fertility following a decline in Ab titres. In another study carried out in C57Bl/6 X A/J F1 mice, it was seen that immunization with a truncated 13 mer from this peptide (330-342 aa residues) along with CFA as an adjuvant led to severe inflammation (Rhim et al., 1992) which was characterized by the presence of lymphocytic infiltrations in the ovary. Activated lymph node cells from the above mice could adoptively transfer oophoritis to naive recipients. By synthesis of truncated peptides, a B cell epitope (336-342 aa residues) overlapping with a T cell epitope (330-338 aa residues) could be identified. CD4+ T cell lines and clones generated against the 13 mer could also induce disease in naive recipients without an Ab response thereby clearly suggesting that ovarian pathology was T cell mediated. In contrast, CD8+ T cells are not required for the induction of oophoritis, suggesting that cell-mediated cytolysis is not involved. Subsequent studies with the 13 mer peptide (330-342 aa residues) indicated that the minimal T cell epitope required for disease induction was localized to the residues 330-337 (NSSSSQFQ). Alanine substitutions along this sequence helped in the identification of residues important either for T cell binding or MHC recognition (Luo et al., 1993; Garza and Tung, 1995). It was also shown that peptide induced T cell mediated oophoritis spontaneously resolves after 4 months and the recovered ovary shows both primordial and developing follicles and no lymphocytic infiltration while being resistant to disease reinduction (Lou et al., 1995a). The chimeric peptide containing the minimal B cell epitope (QFQIHGPR, aa 335-342) with alanine substituting for a T cell cross reactive phenylalanine at position 336, in tandem with a bovine RNAse peptide (aa 94-104) was shown to elicit anti-ZP Abs in mice across 8 different H-2 haplotypes without activation of oophoritic T cells. Thus it was shown that it is possible to overcome MHC
driven non-responsiveness to a self antigen as well as abrogate the T cell response that would be pathogenic to self protein (Lou et al., 1995b). Further studies on autoimmune oophoritis in the mouse model have revealed that autoimmune ovarian pathology as well as auto Ab production induced by immunization with a murine ZP3 peptide (330-340/342 aa residues) previously shown to cause oophoritis in mice could be inhibited by blocking either the gp39 or the CD28 co-stimulatory pathways (Griggs et al., 1996). While it has been suggested that this approach might provide a potential therapeutic strategy to control autoimmune disease, it remains to be seen if inhibition of this pathway also reverses autoimmune oophoritis.

The efficacy of a synthetic peptide approach was also shown in a study in which characterization of polyclonal Abs raised against a panel of synthetic peptides corresponding to hydrophilic regions of pZP3α showed that the peptides were immunogenic, could elicit human zona cross reacting Abs and had in vitro contraceptive efficacy (Hall et al., 1995). Synthetic peptides from the amino terminus of pZP3α and pZP3β conjugated to KLH, were shown to be immunogenic in rabbit. Antiserum against ZP3α and not ZP3β was found to inhibit the binding of boar sperm to pZP (Yurewicz et al., 1993b). The Abs generated in hamsters against a 19-residue peptide from the hZP3 sequence (323-341 aa residues, the homologue of the known linear epitope in mice), reacted with bZP3 as well as hZP (Gupta et al., 1994). The immunogenicity of a hZP3 peptide (327-341 aa residues) was also evaluated in cynomolgous macaques. The peptide was shown to be immunogenic and included both B and T cell epitopes as estimated by peptide specific Ab response as well as a T cell proliferative response. Ovarian steroid metabolite profiles remained normal indicative of unchanged ovarian function. Histological examination of the ovary showed Ig bound to ZP in the follicles of the peptide immunized animals. Sperm binding and acrosome reaction induction functions of these animals remained normal (Mahi-Brown and Moran, 1995). In an extended study, immunization of macaques was done with the macaque homologue of the hZP3 peptide.
used earlier and it was seen that immune response to the peptide was initiated and was accompanied by normal ovarian function (Mahi-Brown, 1996).

**Immunogenicity of Recombinant Antigens**

With the expression of ZP glycoproteins in heterologous expression systems, it became possible to purify sufficient r-ZP antigens for immunization. Initial immunization studies in cynomolgous monkeys using the rabbit rec55 protein and a partial rec75 (rec75a) proteins expressed as cro-β-galactosidase fusion proteins using MDP adjuvant did not elicit a significant Ab response. The proteins conjugated to either protein A or keyhole limpet haemocyanin (KLH) however, generated Abs reactive with the native protein (Schwoebel et al., 1992). Immunization with the rec75 protein produced Abs that interfered with follicular development and ovarian cyclicity, while immunization with the rec55 protein generated Abs that inhibited monkey sperm-egg interaction in vitro and did not affect ovarian follicular development or hormonal profiles (Vande Voort et al., 1995). Immunization of guinea-pigs with glycosylated non-fusion r-rabbit 55 kDa protein expressed in the BEVS generated Abs which inhibited rabbit sperm-egg binding in vitro (Prasad et al., 1996). In another study using r-hZP3, active immunization of marmoset monkeys with purified r-hZP3 resulted in long-term infertility associated with ovarian dysfunction, characterized by suppression of folliculogenesis and depletion of the primordial follicle pool (Paterson et al., 1996).

**Future Directions**

Several laboratories, including ours, are engaged in the expression of ZP glycoproteins, from various species, using a variety of expression systems. These studies will help in delineating more precisely the functional role of ZP glycoproteins during fertilization. Additionally, it will make available r-zona proteins/glycoproteins (without any contamination of ovarian proteins) and allow the undertaking of heterologous or homologous active immunization studies, to study their efficacy to regulate fertility. Novel methods such as using live vaccines for immunization through the oral route or using plants for expression of ZP proteins are also being tried. Another colateral issue of
relevance with respect to the design of a ZP based vaccine will be to delineate the oophoritogenic T cell epitopes so that these may be avoided in the synthetic peptide immunogens. An alternate approach can be to define B-cell epitopes which are immunologically relevant to block fertility and design a synthetic peptide based vaccine incorporating such regions.