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Immunocontraception: Relevance of Zona Pellucida as a Target Antigen

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Introduction

The rising global human population has provided an added impetus to explore new avenues for safe and effective contraceptive methods. An unique experimental approach to reproduction control is that of immunocontraception which entails eliciting an immune response directed against functionally and structurally important antigens that are involved in or produced during mammalian reproduction. Taking fertilization as the focal point of reproduction, blockade by immunocontraception may be pre- or post-fertilization. Antigens specific to the spermatozoa or oocyte are the targets choosen for the former while antigens like hCG, for the later. Antigens used for immunocontraception should be reproduction-specific, highly immunogenic and available in large quantities in a homogenous state. The immune response generated must be capable of reaching the antigen *in situ* and blocking its reproductive function.

In this direction, antigens associated with zona pellucida, the extracellular matrix which surround the mammalian egg, have received considerable attention (Sacco 1987, Paterson & Aitkin 1990). The zona pellucida is comprised of 3-5 families of acidic glycoproteins in most of the species studied(Yurewicz et al. 1986). Expression of ZP is specific to the developing oocyte (Ringuette et al. 1986), and constitute 4 to 8% of the total protein synthesized by the cell in its growth phase(Wassarman 1988). These glycoproteins mediate critical steps in fertilization process including initial attachment followed by tight binding of spermatozoa to the zona pellucida and subsequent activation events associated with it in a relatively species specific manner including prevention of polyspermy. This critical role in reproduction along with their tissue specific nature have made the zona pellucida glycoproteins potential candidate antigens for immunocontraception.

Zona Pellucida— Structural and Functional Aspects

The zona pellucida glycoproteins from different species have been separated by gel electrophoresis and column chromatography. Under non reducing conditions, SDS-PAGE of porcine zona pellucida shows ZP1 (80-90kDa) and ZP3 (55kDa) families (Yurewicz et al. 1987) and human ZP shows ZP1 (90-110kDa), ZP2 (64-78kDa) and ZP3 (57-73kDa) (Shabanowitz & O'Rand 1988). The porcine ZP3 (55kDa) family consists of a mixture of two immunologically and biochemically distinct glycoprotein moieties, each with an apparent molecular weight of 55kDa. These have been identified as ZP3α (core protein 37kDa) and ZP3β (core protein 32kDa). The carbohydrate content of the zona pellucida glycoproteins is 30-40%. Porcine ZP3α has

4 N-linked and 3 O-linked oligosaccharides (OS) while ZP3β has 4 N-linked and 6 O-linked OS chains. The OS are of branched type, rich in sulphated polylactosamines (Noguchi et al. 1992). Purified ZP3α or ZP3β has been obtained following partial enzymatic deglycosylation by endo-β-galactosidase (Yurewicz et al. 1987) or by complete chemical deglycosylation (Henderson et al. 1987a) of ZP3.

The sperm receptor activity is retained in the endo-β-galactosidase treated porcine ZP3 (EBGD ZP3) but lost in the chemically deglycosylated ZP3 (ZP3DG) (Berger et al. 1989). ZP3α (purified by reverse phase HPLC from EBGD ZP3) contributes to a large extent to the sperm receptor activity of ZP3 (Sacco et al. 1989). Testing purified O-linked and N-linked OS from ZP3 shows that O-linked OS from porcine ZP3 can inhibit binding of sperm to oocytes more efficiently than N-linked OS (Yurewicz et al. 1991). The efficiency of this inhibition is far less than that caused by the whole molecule on a molar basis. This suggests that though the carbohydrates might act as substrates for the complementary molecules on sperm (e.g. galactosyl transferase of mouse sperm), the actual recognition might be a function of the positioning of these carbohydrate moieties on the protein backbone which contributes to the spatial conformation recognized as the sperm receptor.

The genes for mouse ZP2 (Liang et al. 1990) and ZP3 (Kinloch et al. 1988), hamster ZP3 (Kinloch et al. 1990), rabbit 55kDa (Schwoebel et al. 1991) and 75kDa (Lee et al. 1993), porcine ZP3α (Yurewicz et al. 1993) and ZP3β (unpublished observations) marmoset ZP3 (Thillai-Koothan et al. 1993a) and human ZP2 (Liang & Dean 1993) and ZP3 (Chamberlin & Dean 1990) glycoproteins have been cloned and sequenced. Porcine ZP3α is the homologue of rabbit rc55 (66% identity) and porcine ZP3β is the homologue of mouse/hamster/human ZP3. **Comparison among the protein sequences of ZP3 genes from different species reveals a 60 to 91% sequence homology. Since fertilization is a species specific event, the regions of maximal differences on the sperm receptor (ZP3), possibly participate in sperm recognition. A hydrophilic domain from residues 322-352 is of maximum disparity among all the species making it a significant region in an otherwise conserved primary structure.**

Both polyclonal and monoclonal antibodies against porcine ZP3 inhibit the binding of boar sperm to porcine oocytes *in vitro* (Sacco et al. 1981, Bagavant et al. 1993a). Polyclonal antibodies to porcine ZP3 and ZP3βDG in addition also inhibit human sperm-egg interaction (Henderson et al. 1987b). A monoclonal antibody specific to porcine ZP4 and ZP1 binds to human zona as studied by indirect immunofluorescence (Koyoma et al. 1991a) and also hinders binding of human sperm (Koyoma et al. 1991b). The antibodies against zona pellucida prevent sperm-egg interaction either by blocking the actual sperm receptor site directly or by preventing the access of sperm to its site of interaction by steric hinderance (Henderson et al. 1988).

Active Immunization Studies

Zona pellucida antigens have been effectively used for immunocontraception in various animal models (Skinner et al. 1984, Mahi-Brown et al. 1985 and Sacco et al. 1987). Immunization of rabbits with the crude zona pellucida preparation leads to irreversible infertility (Skinner et al. 1984). Histology of the ovaries from the immunized animals revealed complete destruction of primordial and developing follicles with formation of cell nests and luteal cysts. Cynomolgus monkeys immunized with crude porcine zona pellucida antigens were also rendered infertile (Gulyas et al. 1983). The irreversibility and abnormal hormonal

profiles associated with this immunization was ascribed to the contamination of granulosa cell processes in the crude zona pellucida preparation. Such a mode of irreversible contraception, though unacceptable to humans will be exploited in controlling animal populations like stray dogs, cats, feral horses (Kirkpatrick et al. 1992) and deer. The use of purified protein fractions were able to alleviate the effect on ovaries to a considerable extent. Sacco et al. (1987) have shown that immunization with purified ZP3 was associated with only a transient alteration of estradiol levels in squirrel monkeys. These animal remained infertile on mating with males of proven fertility. The follicular maturation was normal as shown by the peak progesterone levels in serum.

In our laboratory, female bonnet monkeys, *Macaca radiata*, were immunized with purified 55kDa glycoprotein from porcine zona pellucida (ZP3) and ZP3 conjugated to beta subunit of human chorionic gonadotropin (βhCG) adsorbed on alum. In addition, adjuvants permissible for human use such as nor muramyl dipeptide octylamide (MDP) and sodium phthalyl derivative of lipopolysaccharide have been incorporated in the first injection. Subsequent booster injections included only MDP as an adjuvant. Animals received three doses of either 100 μg of ZP3/injection or 100 μg ZP3 coupled to 125μg βhCG/injection intramuscularly at monthly intervals. Animals including the one receiving ZP3-βhCG conjugate were boosted with 100μg of ZP3 on alum with 1mg of MDP intramuscularly when a decline in anti-ZP3 antibody titres was observed. Animals were allowed to mate with males of proven fertility after the three primary injections and followed up for regulation of fertility for a period spanning upto 2 years. All the animals generated a good anti-ZP3 antibody response as measured by an ELISA. Animals continued to have ovulatory cycles as indicated by the progesterone concentration. Inspite of progesterone concentration indicative of normal ovulation, animals remained infertile in the presence of high anti-ZP3 antibody titres and showed no disturbance in cyclicity. Disturbances observed in cyclicity during May to August in the immunized animals was attributed to summer amenorrhoea as the same was also observable in the non-immunized animals. The disturbance in the cyclicity had no association with high anti-ZP3 antibody titres.

All the animals, except two showed normal circulating basal and peak estradiol concentration estimated before immunization, during high anti-ZP3 antibody titres and following decline in antibody titres. Two animals showed a significant decrease in the peak estradiol levels when the anti-ZP3 antibody titres were high. However, normal peak estradiol concentrations were observed in these two animals subsequent to decline in anti-ZP3 antibody titres. Out of eight immunized animals four conceived after a decline in antibody titres. Three pregnancies continued to term and delivered normal healthy pups. The pups now aged 6 to 12 months are showing a normal growth pattern. One of the pregnant animal aborted during fourth month of gestation. The ovarian morphology of the animals which failed to regain fertility did not reveal any signs of inflammation or lymphocytic infiltrations. Follicles at different stages of developments were observed in all the animals. In two animals the presence of corpus luteum was also observed. No increase in the number of atretic or degenerated follicles was observed. Together these results do not seem to indicate impairment of ovarian function and are therefore promising, but with the small number of animals employed, ovarian dysfunction by ZP3 immunisation cannot be excluded altogether.

Although carbohydrate moieties have been implicated in sperm binding and recognition, antibodies to the protein backbone can also inhibit sperm-egg binding. Rabbits immunized with ZP3DG show a normal ovarian function in terms of follicular development (Jones et al. 1992) and hormonal profiles with concomitant infertility. In primate models, however, ZP3DG in baboons showed decreased levels of estrogen followed by a cessation in ovulation after 8-9 months (Dunbar et al. 1989). ZP3DG in marmoset monkeys protected the animals from pregnancy (Paterson et al. 1992). The hormonal profile and follicular functions of the ovaries were initially normal. Subsequently, the animals went into amenorrhoea. The ovarian morphology showed a gradual depletion of the primordial follicle pool. Immunization in the mouse model with a mouse ZP3 peptide (328 to 342) conjugated to keyhole limpet haemocyanin in random bred NIH Swiss mice caused a long term reversible contraception (Millar et al. 1989) while the same peptide in complete Freund's adjuvant (CFA) given to (C57B1/6 X A/J) F 1 caused severe autoimmune oophorites (Rhim et al. 1992). The oophorites could be transferred to normal adult mice of the same strain by CD4+T cell transfer, proving that the ovarian injury was T cell mediated. By synthesizing truncated peptides in the region it was possible to identify one B cell epitope (336-342) overlapping with a T cell epitope (330-338). The future investigations for a safe peptide vaccine are directed towards identification of peptide stretches of ZP3 from region relevant for fertilization and involving only B cell epitopes excluding area with oophoritogenic T cell activation potential.

Mapping of Functionally Relevant B Cell Epitopes

In order to design an immunocontraceptive vaccine based on synthetic peptides corresponding to functionally relevant B cell epitopes of zona pellucida, murine monoclonal antibodies (MoAbs) having specificity for porcine ZP3α and ZP3β have been developed in our laboratory (Bagavant et al. 1993a, Gupta et al. 1992, 1993 and Chadha et al. 1993). Eleven out of twelve MoAbs against ZP3α recognized ZP3αDG both in ELISA and Western blots suggesting their likely recognition of the protein backbone rather than the carbohydrates. Moreover, seven antibodies reacted with reduced and carboxymethylated form of ZP3α (RCMZP3α) suggesting their likely recognition for sequential epitopes, not sensitive to disulphide reduction. Out of the 13 MoAbs against ZP3β, 10 reacted with ZP3DG and seven reacted with RCMZP3β.

All the MoAbs having specificity for ZP3α or ZP3β reacted with intact zona pellucida of porcine oocytes as revealed by indirect immunofluorescence. Moreover, immunohistochemical localization studies using frozen sections revealed the reactivity only with zona but not with other cellular components of ovarian tissue. None of the antibodies reacted with zonae of mouse and hamster. Various MoAbs reacted to variable extent with zonae of rabbit, dog, goat and monkey. Interestingly 5 antibodies (2 having specificity for ZP3α and 3 having specificity for ZP3β) also reacted with zonae of human. The reactivity of MoAbs with zonae of more than one species suggests that these most likely recognized the conserved sequences.

The efficacy of the various MoAbs to block the binding of ZP3 to sperm was tested in a solid phase enzyme immunoassay. For this purpose biotinylated ZP3 was allowed to bind with sperm membrane vesicles coated on a microtitration plate in the presence or absence of antibodies and bound antigen was revealed by streptavidin-peroxidase conjugate. Polyclonal antibodies raised in rabbits against porcine ZP3 or non-biotinylated ZP3 showed dose

dependent inhibition in the binding of biotinylated ZP3 to boar sperm membrane vesicles. The MoAbs 405, 420 and 28 having specificity for ZP3α and MoAbs 30, 455 and 467 having specificity for ZP3β also inhibited the binding of biotinylated ZP3 to sperm membrane vesicles. Moreover, prior incubation of porcine oocytes with these MoAbs also delayed the zona lysis by trypsin. Interestingly all three antibodies having specificity for ZP3β and two (405 and 420) having specificity for ZP3α recognized the sequential epitopes.

We have previously reported that MoAb 30 also significantly inhibits sperm-zona binding *in vitro* (Bhagavant et al. 1993a). This inhibition is not a function of either antibody affinity or subclass of the MoAb. MoAb 30 having specificity for ZP3β recognizes 14kDa and 6kDa fragments of ZP3β digested with α-chymotrypsin and trypsin respectively. Polyclonal antibodies against the ~6kDa fragment from the tryptic digest of ZP3, reacting with MoAb 30, can also inhibit porcine gamete interaction *in vitro* (Bagavant et al. 1993b). In order to map the determinant recognized by MoAb 30, tryptic digest of ZP3β was resolved on a size column (TSK G2000SW) and small molecular size fractions, immunoreactive with MoAb 30 were pooled, concentrated and further purified to homogeneity on a reverse phase HPLC. The N-terminal amino acid sequence of this fragment was Leu-Met-Glu-Asn-X-X-Ala-Glu, X denoting blank cycles on the analyser. A comparison of the N-terminal sequence of the purified fragment with sequences listed in the Atlas of Protein and Genomic Squences (National Biomedical Research Foundation, USA) shows that this sequence matches with the human ZP3 sequence (Chamberlin & Dean 1990) at amino acids 176 to 184. Whether the two blank cycles obtained in the porcine ZP3β sequence also matched human amino acids 181 and 182 is not known. By using similar strategy, the determinants for MoAbs 467 and 455 have been mapped to first 10 amino acids of N-terminus of ZP3β. Synthetic peptide corresponding to this sequence inhibited the binding of both the antibodies to the native protein in a dose dependent manner.

Conclusions

The use of purified zona pellucida glycoproteins along with permissible adjuvants other than complete Freund's adjuvant have considerably reduced, if not eliminated completely, the alterations in the steroid hormones profile and ovarian pathology. Availability of the amino acid sequence data of various zona proteins and availability of bioeffective MoAbs recognizing sequential epitopes will facilitate the delineation of B cell epitopes which when complexed with the respective antibodies will interfere in sperm-egg interaction. This will be further helped by synthesizing overlapping peptides corresponding to the zona protein amino acid sequences by using multipin peptide synthesis strategy. T-cell help can be provided by coupling these peptides to carrier proteins such as diptheria toxoid or synthetic peptides corresponding to promiscuous T-cell epitopes of malaria antigen (Sinigaglia et al. 1988), amino acid residues 829 to 844 of tetanus toxoid (Panina-Bordingnon et al. 1989) or residues 201 to 222 of diptheria toxoid (Sad et al. 1993). These studies will help in designing zona-based contraceptive vaccine development by employing synthetic immunogens which elicit antibodies that regulate fertility at sperm-zona interaction level without adverse side effects.

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