Contraceptive potential of antibodies to the zona pellucida*

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Summary. The notion of a contraceptive vaccine based on gamete-specific surface antigens was first proposed over a decade ago, as the result of in-vitro and in-vivo studies, and in recent years has been the subject of intensive research. In particular, the zona pellucida has attracted much attention as a potential target for immunological intervention in the fertilization process. Such is the rapidly-expanding nature of research into the biochemical and biological characterization of this structure, that a review of the implications for the development of a contraceptive vaccine seems timely.

Keywords: zona pellucida; contraceptive vaccine; antibodies

Introduction

The global human population continues to grow at an ever-increasing rate, currently by > 220 000 per day. By the end of the century, the world population is forecast to reach 6.3×10^9 , double the figure of 1960, and about 80% of the people will live in Third World countries (Population Statistics Division, OPCS, 1987). It has been widely recognized for some time that new methods of birth control are necessary to halt, and ultimately reverse, this inexorable trend in population growth.

Amongst the various methods of birth control that have been proposed over the past decade or two, arguably the one that has stimulated the greatest interest is that of a contraceptive vaccine (Shivers, 1974). The potential offered by the immunological approach, affording prolonged yet reversible protection against pregnancy, has always made the development of a vaccine highly desirable. In addition to being simple and cheap to make, the use of existing health service infrastructures of Third World nations to administer a vaccine would be an advantage.

An ideal point for the immunological disruption of fertility is the act of fertilization itself. At ejaculation, about 220×10^6 spermatozoa are deposited in the human female reproductive tract, in order to interact with just one other cell—the ovum. The cell recognition events which constitute the fertilization process are, therefore, extremely specific and, for this reason, exhibit some potential as a point of attack for a contraceptive vaccine. There are two such recognition events involved in sperm–ovum interaction, involving the zona pellucida and vitelline membrane of the oocyte respectively, both of which have been shown to be susceptible to immunological interference (Gwatkin *et al.*, 1977; Dor *et al.*, 1981). The zona pellucida, in particular, exhibits promise as the target for a contraceptive vaccine because it possesses tissue (Sacco & Shivers, 1973; Palm *et al.*, 1979) and species-specific (Hanada & Chang, 1972; Hartmann *et al.*, 1972) immunogenic domains which are involved in sperm–ovum interaction. The experiments conducted by Gwatkin & Williams (1976), demonstrating that

*Presented as the Arthur Walpole Memorial Lecture to the Society for the Study of Fertility at the University of York, 1987.

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exposure of capacitated spermatozoa to solubilized homologous zonae pellucidae blocks the ability of the former to interact subsequently with the intact ovum, led to the concept of a zona receptor for spermatozoa, which becomes modified during the post-fertilization block to polyspermy (Oikawa *et al.*, 1975a; Florman & Wassarman, 1985). Subsequent studies have shown the sperm receptor to be extremely sensitive (particularly in the hamster) to protease attack (Hartmann & Gwatkin, 1971), and susceptible to occlusion by lectins (Barros & Yanagimachi, 1972; Oikawa *et al.*, 1975b), polyvalent antibodies (Aitken & Richardson, 1981b) or even univalent (Fab) anti-zona antibody fragments (Sacco *et al.*, 1984). The most detailed characterization of the zona sperm receptor so far has been achieved for the mouse (Florman & Wassarman, 1985) in which this activity has been shown to reside in the oligosaccharide side-chains of a zona glycoprotein of M_r 83 000. The specificity of sperm–zona interaction and the apparent susceptibility of this event to immunological attack have generated considerable interest in the development of anti-zona antibodies as contraceptive agents. The fact that such antibodies would achieve their effect on fertility by blocking conception (rather than the abortifacient mechanism of action associated with alternative approaches to vaccine development, when, for example, hCG is used as the target) also enhances the acceptability of a zona-orientated vaccine programme.

In-vitro studies

The concept of using the zona pellucida as the target for a contraceptive vaccine originated from research, carried out just over a decade ago, on the influence of anti-ovarian antisera on mammalian in vitro fertilization systems (Shivers *et al.*, 1972; Jivek & Pavlok, 1975; Tsunoda & Chang, 1976a, b, 1977). In these studies, it was observed that antisera raised against aqueous extracts of ovarian tissue were able to block fertilization by inhibiting the attachment and binding of spermatozoa to the zona pellucida. It was rapidly established that the ovarian antigens responsible for this inhibitory activity originated from the zona pellucida and that antibodies raised against mechanically isolated zonae *per se* were fully competent to block in-vitro fertilization in laboratory rodents (Tsunoda, 1977; Tsunoda & Chang, 1978).

A major component of the mechanism of action of anti-zona antibodies involves the inhibition of sperm attachment to the zona pellucida by occlusion of sperm binding sites on the zona surface (Aitken *et al.*, 1981). The occlusion of these binding sites is generally thought to involve a process of steric hindrance, rather than direct interaction with the sperm receptor itself. This is particularly true of polyclonal antibodies which, because of their cross-linking activity, are able to induce the formation of an immunoprecipitate (Fig. 1) on the outer zona surface which masks the binding sites for spermatozoa (Aitken & Richardson, 1981b). If the cross-linking activity of anti-zona antisera is destroyed through the preparation of Fab fragments then, in most cases, their abilities to induce the formation of an immunoprecipitate and to block sperm–zona interaction are abolished (Ahuja & Tzartos, 1981; Aitken *et al.*, 1982).

The deposition of an immunoprecipitate on the zona surface is not an absolute requirement for the disruption of sperm–zona interaction, however, since monoclonal anti-zona antibodies (which because of their specificity have a limited capacity for cross-linkage and precipitate formation) are also effective inhibitors of fertilization (Isojima *et al.*, 1984; Koyama *et al.*, 1985; East *et al.*, 1985). This is particularly true when the monoclonal antibodies have been raised against zona antigens from the same species as that used for the in-vitro fertilization assay (Isojima *et al.*, 1984; Koyama *et al.*, 1985; East *et al.*, 1984, 1985). In such circumstances, the monoclonal antibodies are thought to bind to high density antigenic sites on the zona surface which are sufficiently close to the sperm binding site to hinder the latter sterically. Alternatively, even the limited degree of cross-linking achieved with monoclonal antibodies may be sufficient to stabilize the zona pellucida, rendering this structure less susceptible to sperm penetration, even though sperm binding to the zona surface may occur (East *et al.*, 1985).

Fig. 1. Immunoprecipitate formation on the outer surface of the zona pellucida after exposure to anti-zona antibodies. Mouse ova were incubated with polyclonal antibodies raised, in rabbits, against mechanically isolated mouse zonae pellucidae. The presence of an immunoprecipitate on the outer zona surface is indicated by the white arrow.

In contrast, monoclonal antibodies are generally ineffective in disrupting sperm–zona interaction, when the gametes used in the in-vitro fertilization assay are from a heterologous species, even though immunological cross-reactivity may be demonstrable (Isojima *et al.*, 1984; Koyama *et al.*, 1985). In such circumstances, the antigenic site density must be too low for the antibodies to occlude the sperm binding sites or to prevent sperm penetration. However, restoration of the steric hindrance mechanism by adding a second, polyclonal, antiserum directed against mouse immunoglobulin will, in the presence of such monoclonals, lead to immunoprecipitate formation and the disruption of sperm binding in heterologous systems (Koyama *et al.*, 1985). A notable exception is a report of two monoclonal antibodies directed against the pig zona pellucida which were capable of blocking human sperm–zona interaction without the need for a second, cross-linking antibody. One of these monoclonals formed a precipitin line on immunoelectrophoresis with pig zona material and must have been directed against an epitope present as multiple copies on a single antigen molecule, inhibiting sperm–zona interaction by the steric hindrance mechanism described above (Mori *et al.*, 1985). The second monoclonal antibody described in this study did not form a precipitin line during immunoelectrophoresis and suggests the presence of a cross-reactive epitope on the human zona pellucida which is adjacent to, and able to occlude, the sperm binding site (Mori *et al.*, 1985).

Although antisera have been prepared which do appear to interact directly with the sperm receptor (Sacco *et al.*, 1984), the fact that anti-zona antibodies can inhibit sperm–zona interaction by steric hindrance (Aitken & Richardson, 1981b) is absolutely central to any strategy for engineering a contraceptive vaccine. This is because sperm–zona interaction in the human is a highly species-specific event (Bedford, 1977) which is probably mediated by the carbohydrate side-chains on the zona glycoproteins (Florman & Wassarman, 1985). Since it is not currently possible to engineer a vaccine containing defined carbohydrate sequences, it is crucial that antibodies directed against zona peptides *per se* exhibit the ability to interfere with adjacent oligosaccharide sperm-binding sites. The fact that antibodies directed against the polypeptide backbone of zona glycoproteins can still disrupt sperm–zona interaction by interfering with the binding process (Henderson *et al.*, 1987a) or sperm penetration (East *et al.*, 1985) supports the view that antibodies raised against antigens generated by recombinant DNA techniques or direct peptide synthesis should exhibit contraceptive activity *in vivo* and *in vitro*.

In-vivo studies

Passive immunization studies

Passive immunization studies in a variety of species quickly followed the early in-vitro work. It was rapidly established in hamsters (Oikawa & Yanagimachi, 1975), mice (Jilek & Pavlok, 1975) and rats (Tsunoda & Chang, 1976a, b) that passive immunization with antisera against ovarian antigens could induce infertility. Antibodies against cumulus-free mouse eggs or mechanically isolated mouse zonae also proved effective (Tsunoda & Chang, 1978; Sacco, 1979); indeed, prolonged periods of infertility were observed following a single administration of anti-zona antibodies (Tsunoda & Sugie, 1977; Sacco, 1979). Investigation of this last observation revealed that anti-zona antibodies possessed the ability to bind not only to ovulated oocytes, but also to those still in their follicles, with the result that the animal remained infertile for several successive cycles until all ova coated with anti-zona antibodies had been ovulated. Such ova recovered from passively immunized animals were found to possess an immunoprecipitate on the outer surface of the zona pellucida, providing evidence that the mechanism of action of such antibodies *in vivo* was the same as *in vitro*, by sterically hindering the binding of spermatozoa to their receptors on the zona pellucida. Although some workers (Dudkiewicz *et al.*, 1975; Tsunoda & Chang, 1978) have suggested, in the hamster and mouse respectively, that anti-zona antibodies may have effects after fertilization, others have shown an absence of interference with zona shedding and implantation (Tsunoda & Whittingham, 1982) using purified anti-zona antibodies.

The development of monoclonal antibodies against specific zona determinants has further refined this passive immunization approach (East *et al.*, 1984, 1985). Hence, monoclonal antibodies directed against ZP2 (a major component of the mouse zona pellucida which does not include the sperm binding site) have been shown to be extremely effective in preventing the fertilization of mouse ova in passive immunization studies (East *et al.*, 1984). Significantly, this inhibition of fertilization was reversible and did not involve any disruption of folliculogenesis or ovulation. Subsequent studies with monoclonal antibodies directed against ZP3, a sulphated glycoprotein component of the mouse zona pellucida with sperm binding activity, also succeeded in demonstrating a contraceptive effect mediated through the specific disruption of fertilization without any effect on ovarian function (East *et al.*, 1985). These studies confirm the contraceptive potential of anti-zona antibodies *in vivo*. Moreover, they indicate that such efficacy need not involve a direct interaction between the antibody and the sperm receptor site. None of the antibodies utilized by East *et al.* (1984, 1985) were directed against the binding site, and, because of their monoclonal origins, they did not possess sufficient cross-linking activity to occlude these sites by steric hindrance. Simply by binding to the zona pellucida in quantities sufficient to stabilize this structure and prevent sperm penetration to the perivitelline space, such antibodies were extremely effective contraceptive agents.

Active immunization studies

The first example of active immunization employing heterologous zona antigens was provided by Gwatkin *et al.* (1977), who immunized mice with heat-solubilized hamster zonae pellucidae and induced a reversible state of infertility, with no side-effects. However, the ova recovered from these immunized animals possessed 'perforated' zonae pellucidae, the antibodies seriously affecting the integrity of the zonae. Based on the previously demonstrated cross-reactivity between rat and mouse zonae pellucidae (Aitken & Richardson, 1980), and the observation that antibodies against isolated mouse zonae will inhibit fertilization of both mouse and rat eggs *in vitro* (Tsunoda & Chang, 1978; Tsunoda *et al.*, 1981), we carried out a series of experiments in which the duration of infertility after active immunization with heterologous antigen was correlated with the amount of antigen administered (Fig. 2a). The greater the amount of antigen used, the longer the period of

Fig. 2. Anti-zona antibody titre (a) and duration of infertility (b) in the rat after active immunization against mouse zonae pellucidae. Rats were actively immunized against successively larger numbers of mouse zonae pellucidae antigens using Freund's adjuvant. Antibody titre and the duration of infertility were monitored.

infertility induced, until the animals were rendered infertile for what was effectively their reproductive lifespan. Figure 2(b) illustrates the relationship between antigen dose and antibody titre in this model. The infertility induced in such actively immunized rats was invariably dissociated from any adverse side-effects regardless of whether the source of antigen used was an aqueous extract of ovarian tissue (Aitken & Richardson, 1981a), cumulus-free oocytes (Aitken & Richardson, 1981c) or mechanically isolated zonae (D. W. Richardson, unpublished observations). Of particular importance is the fact that the induction of active immunity against the zona pellucida did not influence the normality of ovarian function as indicated by direct measurement of plasma progesterone concentrations or vaginal cytology (D. W. Richardson, unpublished observations). Typically, such immunized animals mated normally and exhibited a series of pseudopregnancies until the antibody titres declined to a point where conception could not be prevented (Fig. 3). Immunized animals not housed with males exhibited an unbroken sequence of normal oestrous cycles (D. W. Richardson, unpublished observations). As further evidence of normal ovarian function immunized animals autopsied on Days 1, 2, 3, 5 or 9 after mating possessed normal numbers of corpora lutea (D. W. Richardson, unpublished observations; Table 1).

Despite the above findings indicating that active immunization results in the inhibition of fertilization without disrupting the processes of follicular growth and ovulation, interference with the latter has been observed when rabbits were immunized with pig zona antigens. In this model, the induction of infertility was associated with the inhibition of normal follicular development and ovulation (Gwatkin *et al.*, 1980; Wood *et al.*, 1981; Skinner *et al.*, 1984), ultimately resulting in the cessation of follicular growth and a reduction in the number of primordial follicles present in the ovary. These data have been interpreted as indicating the destruction of the developing oocyte pool by circulating anti-zona antibodies, when zona antigens are first expressed on the surface of the vitelline membrane (Skinner *et al.*, 1984).

Although this model is of considerable interest in elucidating the role of the oocyte in controlling follicular cell differentiation and possibly the aetiology of certain ovarian diseases, its relevance to the development of a contraceptive vaccine is questionable. The biology of fertilization in the rabbit differs from that observed in other animal models, and the human, in that the zona pellucida only plays a limited role in restricting the passage of spermatozoa into the perivitelline space. As a result, it is normal to find many accessory spermatozoa in the perivitelline space of rabbit ova after

Fig. 3. Induction of infertility in the rat after active immunization against mouse zonae pellucidae. A single experimental profile is shown from one of a group of rats actively immunized against mouse zonae pellucidae using Freund's adjuvant. Antibody titre was monitored by radioimmunoassay and oestrus and mating were detected by vaginal smears.

Table 1. Comparison between corpora lutea numbers in the ovaries of actively immunized and control rats

Days <i>p.c.</i>	Treatment	No. of rats	Mean no. ($\pm$ s.e.m.) of CL per ovary
Day 1	Immunized	10	15.3 $\pm$ 1.21
	Controls	10	14.1 $\pm$ 0.83
Day 2	Immunized	10	13.8 $\pm$ 0.96
	Controls	10	14.3 $\pm$ 0.66
Day 3	Immunized	10	13.9 $\pm$ 0.58*
	Controls	10	10.9 $\pm$ 1.12
Day 5	Immunized	10	13.8 $\pm$ 0.73
	Controls	10	13.4 $\pm$ 0.15
Day 9	Immunized	15	14.4 $\pm$ 0.52
	Controls	15	14.9 $\pm$ 0.68

**P* < 0.05 compared with control value (*t* test).

fertilization *in vivo*. A difference in the physiology of the rabbit zona pellucida is also indicated by the fact that this structure can be extensively cross-linked with lectins to the point at which it cannot be solubilized by proteases and yet spermatozoa can still penetrate through the zona and gain access to the oocyte (Bedford & Cross, 1978). In other eutherian mammals, such cross-linking of the zona pellucida with lectins leads to the inhibition of fertilization through disruption of the sperm–zona interaction (Oikawa *et al.*, 1975b). Most importantly in the present context, exposure of the rabbit ovum to anti-pig zona antibodies *in vitro* does not disrupt the sperm binding process, despite the presence of an immunoprecipitate on the zona surface (Sacco *et al.*, 1981). Such

observations indicate that the physiology of the rabbit zona pellucida is quite different from that observed in most other eutherian mammals and that the mechanisms by which anti-zona antibodies achieve their contraceptive effect are not operational in this species.

One can only speculate as to why the rabbit oocyte should be so susceptible to the cytotoxic effect of anti-zona antibodies, when no such disruption of oocyte viability, and thence folliculogenesis, has been observed in rodent models in active or passive immunization studies. It may be a question of antibody titre. In rabbit studies in which a disruption of ovarian function has been induced large doses of antigen (300–315 µg zona glycoproteins) together with Freund's adjuvant have been used in a model which is notoriously sensitive to the elicitation of humoral immune responses (Wood *et al.*, 1981; Skinner *et al.*, 1984). In contrast, as little as 450 ng mouse zona glycoprotein will induce infertility in an actively immunized rat (Aitken *et al.*, 1984) without any influence on ovarian activity. The active immunization of rabbits with lower amounts of more highly purified pig zona antigens has led to the induction of infertility in rabbits with no adverse side-effects (Skinner & Dunbar, 1984).

Other species used in active immunization studies include the dog (Mahi-Brown *et al.*, 1982, 1985), in which the effects of varying the dose and purity of pig zona antigen, as well as the adjuvant, on hormone profiles and ovarian function were monitored. Again, the major conclusion from this study was that the amount and purity of antigen used in active immunization was crucial to circumvent potential problems with ovarian dysfunction. Adjuvants with the same efficacy as Freund's adjuvant, but with milder physiological side-effects, were also reported.

The cross-reactivity exhibited between primate and pig zonae pellucidae (Sacco *et al.*, 1981, 1983b) led to the use of the latter in a series of active immunization studies with various primate species, and we have studied the common marmoset, *Callithrix jacchus jacchus*. Owing to the well-known intolerance of New World monkeys to Freund's adjuvant, and previous experimental evidence indicating a loss of reproductive function when using Freund's adjuvant in this particular species, alum (aluminium trihydroxide) was used in the active immunization schedules involving these animals. This compound is a much milder adjuvant, necessitating more frequent boosting of the animals in order to maintain antibody levels and infertility. A typical experimental profile from the marmoset study is shown in Fig. 4. In the control group, immunized only with alum, the females became pregnant within 2 weeks of the introduction of a male, whereas those animals actively immunized against the pig zona pellucida (60 µg) remained infertile for a mean period of $47(\pm 15)$ weeks ($N = 3$), with one animal rendered infertile for 75 weeks. Monitoring of plasma progesterone concentrations by radioimmunoassay indicated little disruption to ovarian cyclicity. The experimental animals finally became pregnant when a mating event coincided with a trough of antibody titre, or with an overall lowering of the antibody titre.

Other primate species used in active immunization studies include cynomolgus monkeys (*Macaca fascicularis*), in which Gulyas *et al.* (1983) reported the induction of reversible infertility associated with menstrual irregularities and oocyte atresia, following immunization with pig zonae pellucidae (360 µg in alum). However, the authors stated that, since the zona preparation used in this study was contaminated with non-zona material, they were unable to conclude that such side-effects were due to immunological attack on the zona itself, or whether antibodies against other ovarian components were responsible.

Sacco *et al.* (1983b, 1986a, 1987) have used squirrel monkeys (*Saimiri sciureus*). The immunogen used in these experiments was a purified component of M_r 55 000 of the pig zona pellucida, and a large number of animals (50) was used. After active immunization, high antibody titres were induced in the monkeys for periods up to 18 months, with resultant induction of infertility. Initial disturbances in hormonal status were observed in these animals, along with histological changes suggesting interference in folliculogenesis, although by 10–15 months after immunization hormonal and laparoscopic data indicated a recovery of ovarian function despite continuing high titres of anti-zona antibodies (Sacco *et al.*, 1987). Although the antigen used in this particular experiment was purified, it was administered in relatively high amounts (200 µg), and, in addition, the study used Freund's adjuvant;

Fig. 4. Induction of infertility in the marmoset after active immunization against pig zona pellucida. A single experimental profile is shown from one of a group of marmosets immunized against pig zona pellucida, using alum as adjuvant. Antibody titre was monitored by radioimmunoassay (Hulme *et al.*, 1982). Details of the management of the marmosets and the methods used to monitor their ovarian cyclicity are given by Hearn *et al.* (1975).

the authors acknowledge that the deleterious effects observed on ovarian structure and function may have been entirely due to the latter.

Bonnet monkeys were used by Bamezai *et al.* (1986), and were actively immunized with 200 µg of intact pig zona pellucida with Freund's adjuvant. Again, hormonal changes were observed, with both progesterone and oestradiol concentrations falling during high anti-zona antibody titres, although normal cyclicity was recovered when antibody titres declined. Bamezai *et al.* (1986) stated that moderate immunization against the pig zona pellucida did not irreversibly impair ovarian function and fertility, although they did not appear to consider the possibility of contamination of their zona preparation, or the possible effects of using Freund's adjuvant.

Although more work is necessary in the area of active immunization against the pig zona pellucida, particularly with primate species in properly controlled trials, the studies reported so far encourage continued research into this structure as the basis of a contraceptive vaccine. The overall lesson from these studies is that active immunization schedules should use purified antigen at levels low enough to prevent any side-effects, whilst inducing and maintaining infertility. The results to date support the view that this is an achievable objective.

Fig. 5. Cross-reactivity of human and pig zonae pellucidae demonstrated by indirect immunofluorescence. Salt-stored human ova were incubated for 1 h at 37°C in a polyclonal antiserum raised against intact pig zonae pellucidae, and then further incubated at 37°C for 30 min in a second, fluorescein-labelled antibody raised against rabbit IgG. Immunofluorescence was observed using a fluorescence microscope.

The use of heterologous zona antigens in these, and other, studies is of crucial importance; if homologous antigens were necessary, then the development of a contraceptive vaccine for human use would be seriously hampered by the constraints, both physical and ethical, placed upon the collection of sufficient human zonae pellucidae for experimental purposes. Fortunately, Sacco (1977) has demonstrated that human and pig zonae pellucidae share common antigens, and exhibit cross-reactivity. This is illustrated in Fig. 5, using indirect immunofluorescence with a human ovum and an antiserum raised against components of the pig zona pellucida. The binding of the antibodies to the outer zona surface can be clearly seen and this interaction has been shown to inhibit the subsequent binding of human spermatozoa to the human zona pellucida (Trownson *et al.*, 1980; Hasegawa *et al.*, 1985).

Thus, by the beginning of the decade, attention was turning to the biological and, more particularly, the biochemical characterization of antigens from the pig zona pellucida, in order to produce a basis for the development of a contraceptive vaccine.

The pig zona pellucida

Several methods have been developed for the large-scale isolation of pig zonae pellucidae, most of them using techniques for breaking up the structure of the ovary into successively smaller pieces and releasing the ova and zonae pellucidae from the follicles (Dunbar & Raynor, 1980; Dunbar *et al.*, 1980; Noda *et al.*, 1981; Sacco *et al.*, 1981, 1983a; Hedrick & Wardrip, 1986). We use a commercial meat grinder, passing the ovaries through several times, with different sized cutting screens; details of the protocol are as follows:

- (1) Ovaries removed at slaughter and stored deep frozen
- (2) Ovaries thawed and trimmed of extraneous tissue immediately before processing
- (3) Ovaries successively broken up in meat grinder, copious irrigation with ice-cold saline (0.9% w/v) between passes
- (4) Homogenate passed through 500 and 210 μm nylon meshes to remove large debris
- (5) Filtrate passed through 75 μm nylon mesh to trap eggs and washed off with ice-cold saline
- (6) Centrifuged to concentrate and crude preparation applied to discontinuous Percoll gradient

- (7) Eggs aspirated from top of Percoll gradient, and washed free of Percoll
- (8) Eggs gently homogenized to free zonae, and washed to remove cell contents
- (9) Zonae heat-solubilized (20 min, 76°C)
- (10) Zonae ultracentrifuged, protein content estimated, and zona material lyophilized.

Steps 6 and 7 in this protocol are unique in the area of zonae preparation, and produce an extremely pure preparation, by completely removing potential contaminants. Heat-solubilization of the zonae at the crude oocyte stage results in contamination; by separating the oocytes and zonae by density gradient centrifugation, then using the shearing forces generated in a Potter homogenizer to release the oocytes and another centrifugation step to wash the zonae, we can produce a heat-solubilized preparation consisting only of zona pellucida glycoproteins. In 3 years, we have processed some 25 000 pig ovaries, yielding over 225 mg zona glycoprotein.

Two-dimensional sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) of the pig zona pellucida reveals the presence of 4 glycoprotein families or 'charge-trains', named ZP1-4 in order of decreasing average molecular mass (Fig. 6a). These glycoproteins and their isomers span the molecular mass range from approximately 20 000 to 125 000, and have isoelectric points covering the pH scale from 4 to 8.5 (Dunbar & Raynor, 1980; Dunbar *et al.*, 1981; Sacco *et al.*, 1981, 1983b; Yurewicz *et al.*, 1983; Noda *et al.*, 1983; Hedrick & Wardrip, 1986). The microheterogeneity exhibited by these molecules, residing in the carbohydrate moiety (Hedrick & Wardrip, 1981; Hedrick *et al.*, 1985), is typical of glycoproteins, but is possibly the most extreme example yet known. Although the carbohydrate moieties of the zona glycoproteins are doubtless crucial for the sperm receptor to function (Florman & Wasserman 1985), the current inability to synthesize artificially defined carbohydrate sequences upon which a contraceptive vaccine might be based suggests that further investigation of the polypeptide backbones of the zona glycoproteins is warranted. The major consequence of the charge and molecular mass isomerization exhibited by the zona glycoproteins is the difficulty in isolating any single isomer in sufficient quantity for detailed biochemical analysis. To overcome this obstacle, chemical deglycosylation of the pig zonae glycoproteins has been carried out (Hedrick *et al.*, 1985; Sacco *et al.*, 1986b; Henderson *et al.*, 1987a).

Deglycosylation of pig zonae pellucidae glycoproteins

The methodology used to remove chemically the carbohydrate side-chains from pig zonae glycoproteins involves the use of trifluoromethanesulphonic acid (TFMS) in conjunction with anisole (Karp *et al.*, 1982). We have previously published details of our protocol and subsequent gas chromatographic analysis to demonstrate the complete removal of carbohydrate (Henderson *et al.*, 1987a). The effect of deglycosylation on the electrophoretic profile of the zonae glycoproteins is quite dramatic. After separation on one-dimensional SDS/PAGE, 5 discrete polypeptides can be resolved from the deglycosylated fraction (DGZP), with approximate molecular masses of 66 000 (from ZP1), 52 000 (from ZP2), 36 000, 32 000 (both from ZP3) and 16 000 (from ZP4). These polypeptides are so basic after the removal of carbohydrate that they barely enter the first dimension isoelectric focussing gel when run on two-dimensional SDS/PAGE under the same conditions as the intact zona fraction (ZP). As a result, the polypeptides run as a column of spots in the second dimension (Fig. 6b). Although the component of M_r 32 000 (DG-32) appears to possess residual charge isomerization, this is almost certainly due to molecular changes, such as carbamylation, sulphation or phosphorylation, of the amino acid residues in the polypeptide backbone itself.

Hedrick *et al.* (1985) have also used TFMS treatment to remove carbohydrate, generating polypeptides of approximate molecular masses 71 000, 56 000, 40 000 and 37 000. Taking into account the limits of accuracy expected when calculating molecular masses from electrophoretic mobility, these figures are very similar to those found by our own group. Hedrick *et al.* (1985) also reported that although > 95% of the carbohydrate had been removed, the microheterogeneity was not completely eliminated due to the protein itself being slightly microheterogeneous. Residual heterogeneity following treatment of the pig zona glycoproteins with TFMS has also been reported by Timmons

Fig. 6. Two-dimensional electrophoretic profiles of (a) intact pig zonae pellucidae (ZP) and (b) deglycosylated pig zonae pellucidae (DGZP). Two-dimensional sodium dodecylsulphate/polyacrylamide gel electrophoresis was carried out according to the method of O'Farrell (1975). Samples of ZP and DGZP (Henderson *et al.*, 1987a, b) were prepared for electrophoresis by dissolution in reduced sample buffer, and run in the first dimension in rod gels over the pH range 4–9, and in the second dimension in 10% acrylamide gels over the molecular mass range 10 000–200 000. Polypeptides were visualized by staining with Coomassie blue-R. Residual ampholine staining can be seen at the bottom of each gel. (From Henderson *et al.*, 1987b.)

et al. (1987); although unable to detect carbohydrate by conventional means, these workers showed by lectin blotting that trace amounts of carbohydrate remained on the deglycosylated polypeptides.

TFMS has also been used by Sacco *et al.* (1986b), working with purified ZP3 to generate DG-ZP3, and they have reported that enzymic removal of the carbohydrate side-chains from pig zona

glycoproteins by using endo- β -galactosidase to remove lactosaminoglycans (Yurewicz *et al.*, 1987). After enzymic digestion, ZP3 yielded a pair of partly deglycosylated glycoproteins with approximate masses of 46 000 and 42 000, termed the α - and β -glycoproteins respectively. Several lines of evidence now exist which demonstrate that the ZP3 family from the pig zona pellucida is composed of two sub-families, α and β , based upon polypeptide backbones of molecular masses 32–37 000 and 36–40 000 respectively.

The removal of carbohydrate from the pig zona glycoproteins posed two questions. First, had the immunogenicity of the molecules been affected? In the absence of carbohydrate, would the polypeptide backbones remain immunogenic? Secondly, if antibodies could be produced to the DGZP fraction, would they possess any biological (i.e. contraceptive) activity?

Antibodies to the deglycosylated zona polypeptides

Polyclonal antibodies have been raised to the ZP and DGZP fractions in rabbits (Henderson *et al.*, 1987a), and the question of immunogenicity was answered by using the antiserum to DGZP to probe a two-dimensional immunoblot of intact ZP glycoproteins; the antibodies recognized predominantly the ZP3 family, cross-reacting with all isomers within the charge-train, thus demonstrating that the polypeptide backbones of the ZP glycoproteins retained the ability to induce the formation of antibodies. Sacco *et al.* (1986b) reported that deglycosylated ZP3, whilst retaining significant immunogenic potential, exhibited altered antigenicity after the removal of carbohydrate. These authors were also able to raise polyclonal antisera against the pair of partly deglycosylated glycoproteins of molecular masses 46 000 and 42 000, that were generated by enzymic means from the parental ZP3 family. Furthermore, Wardrip *et al.* (1986) reported the production of monoclonal antibodies against the two fully deglycosylated polypeptides of M_r 37 000 and 40 000, again emphasizing that the polypeptide backbones of the pig zona pellucida glycoproteins were immunogenic in their own right.

To test the contraceptive efficacy of these antisera, and others, we have used an in-vitro human gamete bioassay (Henderson *et al.*, 1987a, b). For this purpose, unfertilized human ova were obtained from in-vitro fertilization programmes and stored in a high-salt medium containing 1.5 M-magnesium chloride and 0.1% dextran (Yanagimachi *et al.*, 1979). This medium has the effect of destroying the oocyte, whilst retaining the structure and function of the zona pellucida. The zonae were removed from this solution and washed in Medium BWB (Biggers *et al.*, 1971), before being preincubated in the antisera under test for 2–3 h at 37°C. The zonae were washed again, and added to 100 μ l droplets under paraffin oil, containing fully capacitated human spermatozoa at a concentration of 10×10^6 ml. After incubation for 16 h at 37°C, the zonae were washed to remove any loosely bound or non-adherent spermatozoa, and then observed microscopically. Relative to a control antiserum (non-immune rabbit serum), antibodies to both the ZP and DGZP fractions completely abolished sperm binding to the zona pellucida (Henderson *et al.*, 1987a), thus demonstrating that the polypeptides of the DGZP fraction possessed antigenic epitopes with contraceptive potential (Fig. 7; Table 2).

Isolation and characterization of DGZP polypeptides

Which of the polypeptides from the DGZP fraction was responsible for the formation of these antibodies with an inhibitory effect on gamete interaction? By using a combination of preparative electrophoresis and electroelution, the deglycosylated polypeptides from the DGZP fraction have been purified to homogeneity on one-dimensional SDS/PAGE (Fig. 8). Polyclonal antibodies have been raised to each of the 4 polypeptides of higher molecular mass, DG-66, DG-52, DG-36 and DG-32 (Henderson *et al.*, 1987b). The effects of these antisera on human gamete interaction are listed in Table 2. Although all the antisera inhibited human sperm binding to the human ZP to a

Fig. 7. Inhibition of human sperm binding to the human zona pellucida by antibodies raised to the intact and deglycosylated pig zona pellucida. Unfertilized human ova were incubated in antisera against the intact and deglycosylated pig zona pellucida before being exposed to capacitated human spermatozoa, according to Henderson *et al.* (1987a, b). Control antiserum was non-immune rabbit serum.

Table 2. Effects of antisera to components of the pig zona pellucida on human gamete interaction

	Antiserum							
	-ve	ZP	DGZP	M_r 32 000	M_r 36 000	M_r 32/36 000	M_r 52 000	M_r 66 000
% spermatozoa bound/100 μm^2 ($\pm$ s.e.m.)	100	0.25 (± 0.14)	1.3 (± 0.06)	5.3 (± 1.2)	22.4 (± 5.8)	9.2 (± 3.5)	61.5 (± 21.6)	70.3 (± 27.8)
No. of expts	6	4	6	5	4	3	2	4

certain degree, the only antiserum which approached the level of inhibition exhibited by the antisera against the ZP and DGZP fractions was that raised against the polypeptide of M_r 32 000, and this inhibited sperm-egg binding by almost 95%.

By using indirect immunofluorescence techniques, the location of the antibody-binding sites can be determined. With antibodies to the ZP and DGZP fractions, and DG-32 (Fig. 9), the binding was localized to the outer surface of the zona pellucida and so the surface architecture of this structure can clearly be seen. Under dark-field illumination, the presence of a well-defined immunoprecipitate can be seen. In contrast, the remaining antisera (anti-DG-52 is used as an example in Fig. 7) failed to form an immunoprecipitate, and their binding to the zona pellucida was more generalized and diffuse throughout the structure. These results underline the importance of immunoprecipitate formation by the antisera in preventing sperm binding to the human zona pellucida.

As a consequence of these studies our major research efforts are now concentrating on the biochemical analysis of the component of M_r 32 000 from the pig zona pellucida as the basis for the

Fig. 8. One-dimensional electrophoresis of polypeptides isolated from the deglycosylated fraction of pig zonae pellucidae. One-dimensional sodium dodecylsulphate/polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). Samples of the polypeptides purified from the DGZP fraction (Henderson *et al.*, 1987b) were prepared for electrophoresis by dissolution in a reduced sample buffer and run on a 10% acrylamide gel covering the molecular mass range approximately 10 000–75 000. Polypeptides were visualized by staining with Coomassie blue-R.

development of a contraceptive vaccine. This work has already begun with analysis of the amino acid composition and N-terminal sequence of DG-32 (unpublished data). Furthermore, we have undertaken the production of monoclonal antibodies to DG-32, and initial results indicate that some of these will bind to the outer surface of the human zona pellucida. As yet, however, we have failed to generate a monoclonal antibody with contraceptive effect; this is in accord with other published reports (Isojima *et al.*, 1984, 1986; Koyama *et al.*, 1985). However, we are persevering with the production of monoclonal antibodies, as the possession of such antibodies will be extremely important in defining the epitopes with contraceptive potential from the pig zona pellucida.

The future . . .

We have devised two parallel strategies for the future research in this programme (Table 3). One involves peptide chemistry, the other the use of recombinant DNA technology; both approaches utilize existing technology and both have the same goal—the elucidation of the primary amino acid sequence of DG-32, which will allow us to make full use of a new technique called epitope mapping (Geysen, 1985; Geysen *et al.*, 1984, 1985). This powerful analytical technique will permit definition of the location of epitopes, with contraceptive potential, from DG-32 to a resolution of a single amino acid, and identify those amino acids within the epitope(s) which are essential for its biological effect. The strength of this method, with the production of a vaccine against the foot-and-mouth disease virus, has been demonstrated (Meloan & Barteling, 1986).

Fig. 9. Visualization of antibody binding to the human zona pellucida by indirect immunofluorescence. Salt-stored human ova were incubated with antisera raised against two polypeptides from the deglycosylated fraction of the pig zona pellucida (DGZP), DG-32 or DG-52 (Henderson *et al.*, 1987b) for 1 h at 37°C. These eggs were then further incubated in a second, fluorescein-labelled, antibody for 30 min at 37°C. Visualization of antibody binding was achieved using a fluorescence microscope (f = fluorescence, d = dark-field conditions). The presence of an immunoprecipitate on the outer surface of the zona pellucida, with antibodies to DG-32, is indicated by the white arrow. No immunoprecipitate is found with anti-DG-52 antiserum.

Once the contraceptive epitopes on DG-32 have been defined, a start can be made on the engineering of a vaccine. Here too, the application of new technology can play a part with the possibility of using genetic engineering techniques to insert the DNA coding for the appropriate zona antigens into *Vaccinia* virus; the altered virus could then be used as a vehicle for the administration of a contraceptive vaccine. Several foreign genes have already been cloned into *Vaccinia* (Keus, 1986), including hepatitis B virus surface antigen (Moss *et al.*, 1984), herpes simplex virus glycoprotein D (Cremer *et al.*, 1985), rabies virus glycoprotein G (Kieny *et al.*, 1984), influenza virus haemagglutinin (Panicali *et al.*, 1983) and malaria sporozoite antigen (Smith *et al.*, 1984). All of these recombinants have been successfully tested in laboratory animals, while the hepatitis recombinant has also been tested in chimpanzees, with the animals rendered resistant to the hepatitis B virus. Alternatively, the possibility exists of using biodegradable depot formulations for the delivery of a contraceptive vaccine (Langer, 1981; Hutchinson & Furr, 1987).

Table 3. Future strategy for the development of a contraceptive vaccine

Peptide chemistry	Recombinant DNA
Chemical/enzymic fragmentation of DG-32	Isolate/sequence N-terminus
Identification of cross-reactive fragments by ELISA/immunoblotting	Synthesize nucleotide probes
Isolation/sequencing of cross-reactive fragments	Prepare cDNA library
	Identify recombinant
	Isolate/sequence insert DNA
	Nick translation
	Screen human genomic library
	Isolate/sequence insert DNA
▼	
Epitope mapping	
▼	
Peptide synthesis	
▼	
Vaccine engineering	
▼	
Active immunization studies	

Vaccines based on synthetic peptides have been formulated against a wide range of bacterial, viral and parasitic infections, including diphtheria (Audibert *et al.*, 1982), cholera (Jacob *et al.*, 1984), hepatitis B (Gerin *et al.*, 1983), influenza (Wilson *et al.*, 1984), poliomyelitis (Emini *et al.*, 1983), malaria (Patarroyo *et al.*, 1987) and HIV (Kennedy *et al.*, 1986). There is no reason to doubt, then, the feasibility of a contraceptive vaccine based on synthetic zona pellucida antigens.

The production of a safe, reliable and effective contraceptive vaccine is still some time away; however, research over the past 15 years has not only established the principle of basing a vaccine on zona-specific antigens, but also put the biochemical basis for the development of such a vaccine within our grasp.

C.J.H. is the holder of an MRC/Celltech Training Fellowship. We thank Professor A. A. Templeton, Department of Obstetrics and Gynaecology, University of Aberdeen, Dr V. J. Bolton, IVF Unit, King's College, London, Dr P. Braude, Department of Obstetrics and Gynaecology, University of Cambridge, for assistance and co-operation; and Hall's Ltd, of Broxburn, West Lothian, U.K. for the supply of pig ovaries.

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Received 14 September 1987