Recent advances in contraceptive vaccine development: a mini-review

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Contraceptive vaccines (CV) may provide viable and valuable alternatives to the presently available methods of contraception. The molecules that are being explored for CV development either target gamete production [luteinizing hormone-releasing hormone (LHRH)/GnRH, FSH], gamete function [sperm antigens and oocyte zona pellucida (ZP)], and gamete outcome (HCG). CV targeting gamete production have shown varied degrees of efficacy; however, they either affect sex steroids causing impotency and/or show only a partial rather than a complete effect in inhibiting gametogenesis. However, vaccines based on LHRH/GnRH are being developed by several pharmaceutical companies as substitutes for castration of domestic pets, farm and wild animals, and for therapeutic anticancer purposes such as in prostatic hypertrophy and carcinoma. These vaccines may also find applications in clinical situations that require the inhibition of increased secretions of sex steroids, such as in uterine fibroids, polycystic ovary syndrome, endometriosis and precocious puberty. CV targeting molecules involved in gamete function such as sperm antigens and ZP proteins are exciting choices. Sperm constitute the most promising and exciting target for CV. Several sperm-specific antigens have been delineated in several laboratories and are being actively explored for CV development. Studies are focused on delineating appropriate sperm-specific epitopes, and increasing the immunogenicity (specifically in the local genital tract) and efficacy on the vaccines. Anti-sperm antibody (ASA)-mediated immunoinfertility provides a naturally occurring model to indicate how a vaccine might work in humans. Vaccines based on ZP proteins are quite efficacious in producing contraceptive effects, but may induce oophoritis, affecting sex steroids. They are being successfully tested to control feral populations of dogs, deer, horses and elephants, and populations of several species of zoo animals. The current research for human applicability is focused on delineating infertility-related epitopes (B-cell epitopes) from oophoritis-inducing epitopes (T-cell epitopes). Vaccines targeting gamete outcome primarily focus on the HCG molecule. The HCG vaccine is the first vaccine to undergo Phase I and II clinical trials in humans. Both efficacy and lack of immunopathology have been reasonably well demonstrated for this vaccine. At the present time, studies are focused on increasing the immunogenicity and efficacy of the birth control vaccine, and examining its clinical applications in various HCG-producing cancers. The present article will focus on the current status of the anti-sperm, anti-ZP, anti-LHRH/GnRH and anti-HCG vaccines.

Key words: birth control/contraceptive vaccine/oocyte/hormones/sperm

Introduction

Besides the availability of the present methods of birth control, the population explosion and unintended pregnancies continue to pose major public health issues worldwide. The world population has exceeded 6.43×10^9 (World POPClock projection, 2005) and increasing by 1×10^9 every 12 years. Ninety-five percent of this growth is in the developing nations. In the USA, half of all pregnancies are unintended, which result in $>1 \times 10^6$ elective abortions annually (Henshaw, 1998; Grow and Ahmed, 2000). This calls for a better method of contraception that is acceptable, effective and available both in the developed and developing nations. An ideal contraceptive method should be highly effective and safe, inexpensive, have a prolonged duration of action, be rapidly reversible

and easily accessible, require infrequent administration, and be capable of private use (Contraception Online, 2004). A contraceptive vaccine (CV) has been proposed as a valuable alternative that can fulfil most, if not all, of the properties of an ideal contraceptive. Since the developed and most of the developing nations have an infrastructure for mass immunization, the development of vaccines for contraception is an exciting proposition.

Discussion

Several targets are being investigated in various laboratories for the development of CV. These can be divided into three main categories: CV targeting gamete production, gamete

function and gamete outcome (Naz, 2005a). CV targeting gamete production include anti-luteinizing hormone-releasing hormone (LHRH)/GnRH vaccines; those targeting gamete function include anti-sperm and anti-oocyte zona pellucida (ZP) vaccines; and those targeting gamete outcome include anti-HCG vaccines. The aim of this article is to review the progress on various CV that has been made during the last 5 years, their current status, limitations (if any), and future perspectives. Specifically, this article will focus on anti-sperm, anti-ZP, anti-LHRH/GnRH, and anti-HCG vaccines.

Anti-sperm vaccine

Anti-sperm vaccine represents an exciting proposition for contraception and has drawn a considerable interest. The rationale and feasibility for the development of a sperm vaccine are strong and convincing. Sperm have both auto- and isoantigens, and can therefore produce antibodies in both men and women. Anti-sperm antibodies (ASA) affect fertilization and fertility both *in vitro* and *in vivo*. The presence of ASA in the IVF medium blocks fertilization. Deliberate immunization of males or females of various species (Edwards, 1964), including humans (both women and men), with sperm or their extracts causes the development of ASA leading to infertility (Baskin, 1932). Up to 70% of vasectomized men produce ASA (Liskin *et al.*, 1983) and 2–30% of cases of infertility may be associated with the presence of ASA in the male and/or female partner of an infertile couple (Ohl and Naz, 1995). Thus, sperm can generate an immune response that is capable of inducing a contraceptive state. However, the whole spermatozoon *per se* cannot be used for the development of a vaccine because of the presence of several antigens that are likely to be shared with various somatic cells (Naz, 1999). Only those antigens that are sperm specific can be employed for CV. The application of a sperm antigen in a CV is contingent upon its sperm specificity, surface expression, involvement in fertility, and ability to raise enough antibodies to be capable of intercepting fertility. If an antigen is also involved in human immunoinfertility, it is an especially attractive candidate. The sperm–ZP binding site constitutes the most attractive target for immunocontraception.

Several laboratories, including ours, have been using the hybridoma and recombinant DNA technologies, and various proteomic and genomic approaches, to search for sperm-specific antigens that can be used for CV development. It is envisaged that if one obtains the right type of antigen, it will help to construct an ideal anti-sperm vaccine. Several antigens have been identified. Some of them have been further characterized and the complementary DNA (cDNA) encoding these antigens have been cloned and sequenced. Notable among them are the fertilization antigen (FA)-1 (Naz, 1999), PH-20 (Primakoff *et al.*, 1988), PH-30 (Hardy *et al.*, 1997), sperm protein (SP-10) (Herr *et al.*,1990), SP-17 (Lea *et al.*, 1998), testis-specific antigen-1 (Santhanam and Naz, 2001), contraceptive vaccinogen (Naz *et al.*, 2001), protein A-kinase anchoring protein (AKAP) (Miki *et al.*, 2002), and sperm-associated antigen 9 (SPAG9) (Shankar *et al.*, 2004). The list is growing very fast. Gene knock-out technology has provided a potent tool for delineating several sperm molecules that might have a role in sperm

development, structure and function. However, it needs to be carefully evaluated how many of these proteins indeed meet the criteria required for the CV development. We did a careful analysis of the gene knock-out mice that have been published in the literature and which have been shown to have a defect in fertility (Naz and Rajesh, 2005). Although several new exciting genes/proteins have been found that have a role in spermatogenesis, sperm motility, acrosome/capacitation or fertilization cascade, and could provide interesting targets for pharmacological inhibition, most of them are not amenable to immunoneutralization by antibodies. Thus, they may not be suitable candidates for CV development.

Active immunization of female animals with some of these antigens (FA-1, PH-20 and SP-17) has been shown to reduce fertility *in vivo*. To date, no single antigen has been shown to cause a 100% reduction in fertility in the mouse model, a commonly used model for testing the efficacy of a contraceptive vaccine. The maximum effect so far obtained is up to 75% reduction using potential toxic and non-permissible adjuvants such as Freund's adjuvant. This may be due to: (i) the inherent nature of the model—it may be difficult/impossible to render mice completely infertile; (ii) multiple antigens are involved in the fertilization cascade; and (iii) vaccination with no single antigen has raised enough antibody titre, especially in the local genital tract, to completely block fertility. Research in several laboratories is currently focused on answering these questions. It seems that no single antigen will be able to provide 100% protection in all subjects. The research from our laboratory showed that in humans there are at least four sperm proteins involved in oocyte ZP binding (Naz and Ahmad, 1994). A similar situation seems to exist in the mouse model. At this time, there is no published report examining the contraceptive effect of more than one sperm antigen in a single vaccine formulation in any animal model. It is hypothesized that the immunization with multiple fertility-related antigens will increase the efficacy of a vaccine by an additive effect unless these antigens have an immunosuppressive effect on each other's immunogenicity. However, it is interesting to note that several studies have observed a complete block of fertility in a few mice after immunization with a single antigen. It is possible that these animals develop a high cell-mediated immune (CMI) response, besides antibody response, that has a bystander deleterious effect on sperm/oocyte/embryo function. Several cytokines such as tumour necrosis factor (TNF)-α and γ-interferon have deleterious effects on sperm and embryos (Naz *et al.*, 2000a) and immunization with several antigens does cause induction of CMI response and production of such cytokines (Naz and Mehta, 1989). Thus, to enhance the efficacy of a vaccine, it may be important to induce both the CMI as well as humoral immune responses. DNA vaccination may provide such an alternative. At this time, a DNA vaccination approach has not been examined for a sperm antigen. However, in other systems, it has been reported that DNA vaccination favours memory and CMI response rather than effector B-cell response (Laylor *et al.*, 1999).

One can speculate that since a mouse ovulates several oocytes and a woman normally ovulates one oocyte during each cycle, the 75% reduction in fertility by a sperm antigen in

the mouse model may be equivalent to 100% reduction in humans. Only a few studies have examined the effect of vaccination based on a sperm antigen on fertility in a non-human primate model. Vaccination with testis-specific lactate dehydrogenase (LDH-C$_4$) reduced fertility in female baboons (O'Hearn *et al.*, 1997). However, a study by another group reported no effect on fertility in female monkeys (Cynomolgus macaque) after vaccination with LDH-C$_4$ (Tollner *et al.*, 2002). The reason for this discrepancy is not clear. Recently, in an interesting study, male monkeys (*Macaca radiata*) were immunized with an epididymal protein, designated as epididymal protein inhibitor (Eppin) (O'Rand *et al.*, 2004). After immunization, 78% (7/9) monkeys developed high antibody titres to Eppin and became infertile, and 71% of them recovered fertility after immunization was stopped. To maintain high antibody titre, booster injections with Freund's adjuvant have to be given every 3 weeks for almost the whole study duration of 691 days. The potential immunopathological effects of immunization were not examined. This study indicates that anti-sperm CV can also be used for men.

To conduct Phase I and II multicentre clinical trials in a quality-controlled manner, the antigens have to be either recombinant or synthetic molecules. The peptide-based vaccines have some advantages over using the whole recombinant antigens. The peptides are well-defined molecules that could be synthesized and purified in large quantities at relatively lower cost than the recombinant antigens. Several synthetic sperm peptides have also been investigated for contraception. Vaccination with sperm peptides has caused various degrees of contraceptive effects in animal models (Lea *et al.*, 1998; Hardy and Mobbs, 1999). Recently, using phage display technology we identified a novel dodecamer peptide sequence, YLPVG-GLRRIGG, on human sperm, designated as YLP$_{12}$, that is involved in binding to the complementary molecule, human oocyte ZP3 (Naz *et al.*, 2000b). Since human oocyte ZP, which is glycosylated, was used as a probe to identify the YLP$_{12}$ sequence, it is possible that it is a peptide mimetic of the carbohydrate moiety present on sperm that is involved in ZP binding.

A vaccine was prepared by conjugating the synthetic YLP$_{12}$ peptide with recombinant cholera toxin B subunit (rCTB) (Naz and Chauhan, 2002). Since YLP$_{12}$ peptide is only 12-mer long, rCTB was used to provide T-cell help to make the vaccine immunogenic. CTB has been successfully used both as a carrier and as an adjuvant for enhancing systemic and genital tract immunity. Vaccination of female mice with the YLP$_{12}$-rCTB conjugate by various routes (intranasal/intramuscular) produced a sperm-specific immune response inducing a contraceptive state, causing a significant reduction (up to 71%) in litter size. All vaccinated animals showed some degree of inhibition of fertility, and the animals with high antibody titres, both in sera as well as vaginal washings, showed a complete block. By days 305–322 the antibodies completely disappeared from serum and vaginal washings of the vaccinated animals. Upon mating, the antibody-free animals delivered a normal litter size, indicating a restoration of fertility. It was also investigated whether the contraceptive effect could be reversed voluntarily before the effect of the vaccine subsides due to disappearance of antibodies over time. Administration of the YLP$_{12}$ peptide by the intravaginal route, to neutralize the antibodies, resulted in the restoration of fertility. Peptide administration did not cause a booster effect on the antibody titres. These data indicate that the contraceptive state after anti-sperm vaccination could be reversed voluntarily at a given time if desired.

Besides utility in CV development, the sperm antigens will also have clinical applications in the specific diagnosis and treatment of infertility. The FA-1 antigen has been shown to be associated with immunoinfertility in men and women (Naz, 1999). A clinical trial was conducted at the University of Michigan Medical School (Ann Arbor, MI, USA) to determine whether immunoadsorption with the FA-1 antigen would remove autoantibodies and increase the amount of antibody-free sperm. Incubation of sperm from immunoinfertile men with FA-1 antigen removed the autoantibodies and increased the amount of antibody-free sperm, resulting in a significant increase in acrosome reaction rates. The intrauterine insemination of FA-1 antigen-adsorbed antibody-free sperm resulted in normal pregnancies and healthy babies, indicating that the antigen treatment does not have deleterious effects on implantation or on embryonic and fetal development. This study is being extended to a larger number of immunoinfertile men for immunotherapeutic purposes (Menge *et al.*, 1999).

The presence of antibodies against an antigen in infertile men and women is very interesting since it indicates: (i) association of these antibodies and antigens with human infertility; (ii) immunogenicity of these antigens in humans; and (iii) since most infertile men and women are healthy individuals without any disease concomitant with infertility, the presence of antibodies to an antigen is indicative, though not confirmatory, of sperm specificity. Thus, if an antigen is involved in human immunoinfertility, extensive Phase I clinical trails to investigate the immunopathological consequences in actively immunized subjects may not be absolutely necessary. These immunoinfertile men and women provide involuntarily vaccinated models that illustrate how a CV based on sperm antigens will work in humans. There has been a lot of interest in delineating those antigens that are involved in ASA-mediated immunoinfertility (Auer *et al.*, 1995; Naz, 2005b; Pillai *et al.*, 1996). However, only a few antigens have been identified that have been shown to be clearly associated with human immunoinfertility.

Anti-ZP vaccine

During fertilization, zona pellucida (ZP) matrix that surrounds the mammalian oocyte plays a critical role in the recognition and binding of sperm to the oocyte, induction of acrosomal exocytosis in the zona-bound sperm, and avoidance of polyspermy. ZP is primarily composed of three glycoproteins designated as ZP1, ZP2 and ZP3, based on their mobility in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). However, ZP glycoproteins isolated from various species revealed variable mobility on SDS–PAGE, which is primarily due to the differences in post-translational modifications, including glycosylation, in spite of having a very similar polypeptide core. To avoid any ambiguity, an alternative

nomenclature of ZPA (ZP2), ZPB (ZP1) and ZPC (ZP3) has been proposed, based on the size of the transcript (Harris *et al.*, 1994). According to this classification, ZPA is encoded by the longest transcript and ZPC by the smallest transcript. However, recent studies revealed that human ZP is composed of four glycoproteins designated as ZP1, ZP2, ZP3 and ZPB (Lefievre *et al.*, 2004). Comparison of the deduced amino acid (aa) sequences of the cDNA clones for the ZP glycoproteins from different species revealed variable degrees of conservation (Table I). Thus, the antibodies generated against ZP glycoprotein of a particular species exhibit immunological cross-reactivity with the ZP from several other species. This property of ZP glycoproteins has permitted heterologous immunization strategies to achieve contraceptive efficacy.

Immunocontraceptive potential of ZP glycoproteins isolated from native source

Due to their easy accessibility from abattoirs, and immunological cross-reactivity with the ZP of various species including humans, porcine ZP glycoproteins became the antigens of choice. Initial studies of active immunization of female rabbits and monkeys with porcine heat-solubilized isolated ZP (SIZP) revealed the efficacy of this procedure to achieve infertility (Wood *et al.*, 1981; Gulyas *et al.*, 1983). The resulting infertility was not due to the interference at the level of sperm–oocyte interaction but due to follicular atresia and accompanying abnormal hormonal profile. At this juncture, it was argued that the porcine SIZP employed in these studies may be contaminated with other ovarian-associated proteins, which may be responsible for observed ovarian dystrophy. Subsequent active immunization studies in non-human primates with purified porcine ZP glycoproteins demonstrated curtailment of fertility in the immunized animals with reduced adverse effects on ovarian functions (Sacco *et al.*, 1987; Bagavant *et al.*, 1994). In addition to the purity of ZP glycoproteins, the nature of adjuvants employed in active immunization studies was also shown

to influence the efficacy as well as the safety of the procedure (Sacco *et al.*, 1989; Upadhyay *et al.*, 1989).

Recombinant ZP proteins as an alternative to native ZP glycoproteins

Limited amounts of ZP glycoproteins can be obtained from native sources. Further, there may be batch-to-batch variations in the quality of the purified product, besides a risk of contamination with the other ovarian-associated proteins. To circumvent the above problems, various research groups have produced recombinant ZP proteins and evaluated their efficacy to inhibit fertility. Long-term infertility was observed in female marmosets (*Callithrix jacchus*) immunized with recombinant human ZP3 expressed in mammalian cells (Paterson *et al.*, 1998). However, the observed infertility was associated with ovarian pathology characterized by depletion of primordial follicles. In another study, a comparative immunocontraceptive efficacy of recombinant human ZP1, ZP2 and ZP3 expressed in Chinese Hamster Ovarian (CHO) cells was evaluated in two different non-human primate species namely cynomolgus monkeys (*Macaca fascicularis*) and baboons (*Papio cynocephalus*) (Martinez and Harris, 2000). Both cynomolgus monkeys and baboons immunized with ZP1 remained infertile ranging from 9 to 35 months. During the time of high antibody titres, some immunized animals experienced disruption of the menstrual cycle, but eventually all the animals resumed normal menstrual cycles. Control animals and animals immunized with recombinant human ZP2 and ZP3 conceive before any of the recombinant human ZP1-immunized animals, suggesting that ZP1 is a better candidate for curtailment of fertility as compared to ZP2 and ZP3 (Martinez and Harris, 2000). In another study, female baboons (*Papio anubis*) were immunized with the *E. coli*-expressed recombinant bonnet monkey (*Macaca radiata*) ZP1 (bmZP1) conjugated to diphtheria toxoid (DT) (Govind and Gupta, 2000). Immunization led to generation of high antibody titres against ZP1 and immunized animals continued to exhibit normal ovulatory cycles in spite of high circulating antibody titres. Immunized animals, during the period of high antibody titres ($>2\times10^3$ antibody units), failed to conceive when mated with males of proven fertility. However, subsequent to decline in antibody titres, the immunized animals became pregnant upon mating. Employing a homologous non-human primate model, female bonnet monkeys were immunized with the *E. coli*-expressed recombinant bmZP1 and bonnet monkey ZP2 (bmZP2) conjugated independently with DT (Govind *et al.*, 2002). Analysis of the antibody titres in the respective groups of immunized female monkeys revealed high circulating antibodies against ZP1 and ZP2. The immunized animals showed protection from conceiving for cumulative 45 ovulatory cycles in the ZP1-immunized group and 32 ovulatory cycles in the ZP2-immunized group. Both groups of immunized animals failed to conceive even after the decline in the antibody levels. Ovarian histology of the immunized animals revealed the presence of atretic follicles with degenerating oocytes, which may explain failure to conceive even when the antibodies were not detectable in the circulation.

Table I. Sequence identity at the deduced amino acid (aa) level of the three zona pellucida (ZP) glycoproteins from various species with their respective human homologues

ZP family	Species	Length of polypeptide chain (aa)	% identity with human
ZP1	Mouse	622	39
	Rabbit	533	71
	Pig	533	68
	Bonnet monkey	539	92
	Human	565	–
ZP2	Mouse	713	60
	Rabbit	672	72
	Pig	716	64
	Bonnet monkey	745	94.2
	Human	745	–
ZP3	Mouse	424	67
	Rabbit	415	69
	Pig	420	74
	Bonnet monkey	424	93.9
	Human	424	–

In future, it may be worth exploring the plausible reasons why active immunization with recombinant bmZP1 in a heterologous animal model (baboon) led to reversible block of fertility in contrast to irreversible block of fertility generated in a homologous animal model (bonnet monkey). Is it due to qualitative differences in the antibody responses generated in the two species or greater susceptibility of bonnet monkeys to develop oophoritis? Another study suggested that the nature of glycosylation of the recombinant zona proteins may also influence their immunocontraceptive efficacy (Hardy *et al*., 2003). Female BALB/c mice immunized with recombinant mouse ZP3 produced in mammalian cells exhibited a reduction in fertility, whereas those immunized with baculovirus-expressed mouse ZP3 remained fertile.

Potential of ZP glycoprotein-based contraceptive vaccine for controlling populations of wild and domestic animal species

It is imperative to develop new strategies for the management of wildlife populations in a humane way, as many cultures forbid hunting and killing of animals. Is a contraceptive vaccine based on ZP glycoproteins that is meant only for the female population a viable proposition? It is anticipated that such a vaccine with 50–70% efficacy will still be effective in controlling wildlife populations. In feral horses (*Equus caballus*), a single annual booster of porcine ZP antigens was enough to prevent conception, without affecting the complex social behaviour of the immunized animals (Turner *et al*., 2002). Short-term treatment for up to 4–5 consecutive years did not result in any detectable debilitating side-effects and the contraceptive effect was reversible, whereas long-term treatment for 7 consecutive years has not resulted in return in fertility (Kirkpatrick and Turner, 2002). There was no difference in survival rates between foals born to treated and untreated mares, and vaccination of pregnant mares did not affect subsequent fertility of their female offspring. Immunization of white-tailed deer (*Odocoileus virginianus*) with native porcine ZP antigens also caused a significant decrease in the fawning rates (Miller *et al*., 1999). Subsequently, it was demonstrated that this approach is useful in maintaining white-tailed deer population density and composition living on Fire Island, New York, USA (Naugle *et al*., 2002).

This approach also works in African elephant (*Loxodenta africana*) (Fayrer-Hosken *et al*., 1999) but failed to prevent fertility in domestic kittens (*Felis catu*) (Gorman *et al*., 2002). The failure to reduce fertility in cats was due to non-reactivity of antibodies against porcine ZP antigens to feline ZP. To evaluate the contraceptive potential of ZP glycoproteins in dogs, female dogs were immunized with *E. coli*-expressed recombinant dog ZP3 and ZP2 conjugated to DT, which resulted in the curtailment of fertility in ZP3-immunized animals (Srivastava *et al*., 2002).

Alternative mode of vaccine delivery

The effective vaccine delivery will be a key component, if this approach is to be used for management of the free-ranging wild animal population. In this direction, a porcine ZP antigen-based contraceptive vaccine has been made into pellets, which can fit into the needle of a dart or a syringe, thus making remote delivery a feasible proposition (Turner *et al*., 2002). A single injection of porcine ZP antigens encapsulated in liposomes produced a long-lasting contraceptive effect in grey seals (*Halichoerus grypus*) (Brown *et al*., 1997). Partial contraceptive efficacy has been reported in mice immunized orally with attenuated *Salmonella typhimurium* expressing recombinant murine ZP3 (Zhang *et al*., 1997). In an additional approach, host-specific live vectors have been deployed to deliver zona proteins. Immunization of mice with recombinant ectromelia virus (a natural pathogen of mice that causes mouse pox) and murine cytomegalovirus (mouse-specific beta herpes virus expressing mouse ZP3) resulted in curtailment of fertility (Jackson *et al*., 1998; Lloyd *et al*., 2003). In similar vein, the contraceptive potential of recombinant myxoma virus expressing rabbit ZP1 in female rabbits has also been reported (Gu *et al*., 2004).

The potential of DNA vaccine in the context of ZP glycoprotein-based contraceptive vaccines has also been addressed. Immunization of mice with DNA vaccine encoding bmZP1 in saline generated antibodies, which inhibited *in vitro* the binding of human sperm to zona in the hemizona assay (Rath *et al*., 2002). DNA vaccine encoding canine ZP3 also generated antibodies in mice reactive with native canine ZP (Rath *et al*., 2003). The *in vivo* contraceptive efficacy of these two constructs is still awaited. In another study, immunization of mice with the plasmid DNA encoding partial sequence of rabbit ZP3 (aa residues 263–415) led to inhibition of fertility without any disturbances in folliculogenesis (Xiang *et al*., 2003).

Design of synthetic peptides corresponding to ZP glycoproteins with immunocontraceptive potential

Employing a series of elegant experiments, it was demonstrated that the 'oophoritogenic' T-cell epitopes present in the zona proteins may be responsible for ovarian dysfunction often observed after immunization with ZP antigens (Lou *et al*., 1993). Hence efforts were made by several research groups to delineate the B-cell epitopes of the three ZP glycoproteins that are devoid of 'oophoritogenic' T-cell epitopes (Hinsch *et al*., 1998; Sun *et al*., 1999; Hasegawa *et al*., 2002; Sivapurapu *et al*., 2002; Ringleb *et al*., 2004). Table II summarizes some of the potential synthetic peptides corresponding to ZP glycoproteins. The feasibility of this approach for immunocontraception was demonstrated by active immunization of mice of eight different haplotypes and B6AF1 mice. The immunization with chimeric peptide comprised of 'promiscuous' T-cell epitopes of bovine RNase (NCAYKTTQANK), co-linearly synthesized with the minimal B-cell epitope of mouse ZP3 (335–342 aa; Phe_{336} substituted by Ala) led to curtailment of fertility without ovarian dysfunction (Lou *et al*., 1995).

No disruption of ovarian function was observed following immunization of female marmosets with synthetic peptide corresponding to human or marmoset ZP3 in contrast to recombinant protein (Paterson *et al*., 1998, 1999). Immune sera from animals immunized with marmoset ZP3 peptide (301–320 aa) significantly reduced the *in vitro* binding of human sperm to human zona. However, *in vivo* studies did not show consistent

Table II. Zona pellucida glycoprotein-based synthetic peptide immunogens

Synthetic peptide	Species immunized	Outcome of active immunization	Reference
Bonnet monkey ZP1 (251–273 aa)	Mouse	Antibodies inhibit *in vitro* human sperm–oocyte binding	Sivapurapu *et al.*, 2002
Porcine ZP1 (79–130 aa)	White-tailed deer	Curtailment of fertility	Miller and Killian, 2002
Feline ZP1 (130–149 aa)	Rat	Antibodies inhibit *in vitro* cat sperm–oocyte binding	Ringleb *et al.*, 2004
Human ZP2 (541–555 aa)	Rabbit	Antibodies inhibit *in vitro* human sperm–oocyte binding	Hinsch *et al.*, 1998
Human ZP2 (50–67 aa)	Rabbit	Antibodies inhibit *in vitro* human sperm–oocyte binding	Hasegawa *et al.*, 2002
Mouse ZP2 (121–140 aa)	Mouse	Block in fertility without oophoritis	Sun *et al.*, 1999
Bonnet monkey ZP3 (334–343 aa)	Mouse	Antibodies inhibit *in vitro* human sperm–oocyte binding	Sivapurapu *et al.*, 2002
Bonnet monkey ZP3 (324–347 aa)	Bonnet monkey	Block in fertility, no disruption of cyclicity, normal folliculogenesis	Kaul *et al.*, 2001
Marmoset ZP3 (301–320 aa)	Marmoset	Normal ovarian function, antibodies showed *in vitro* contraceptive efficacy	Paterson *et al.*, 1999
Mouse ZP3 (335–342 aa) Phe$_{336}$ replaced by Ala	Mice of eight different haplotypes	Block in fertility without concomitant oophoritis	Lou *et al.*, 1995
Mouse ZP3 (328–342 aa)	Wild mouse	Curtailment of fertility	Hardy *et al.*, 2002

reduction in fertility (Paterson *et al.*, 1999). Female bonnet monkeys immunized with bonnet monkey ZP3 peptide (324–347 aa) conjugated to DT failed to conceive despite having ovulatory cycles (Kaul *et al.*, 2001). No ovarian pathology was observed in the immunized animals. Immunization of white-tailed deer with porcine ZP1 peptide (79–130 aa; Miller and Killian, 2002) and wild mice with mouse ZP3 peptide (328–342 aa; Hardy *et al.*, 2002) also led to significant reduction of fertility.

These observations still keep the hopes alive for ZP glycoproteins based contraceptive vaccines for human use. In general, peptides as compared to proteins are less immunogenic. To enhance the immunogenicity and hence contraceptive efficacy of peptide-based vaccines, chimeric immunogens encompassing multiple epitopes of zona proteins have been proposed. Synthetic peptide comprising the B-cell epitopes of bmZP1 (251–273 aa) and bmZP3 (324–347 aa) separated by a tri-glycine spacer produced antibodies having higher *in vitro* contraceptive efficacy as compared to the antibodies generated against individual peptides (Sivapurapu *et al.*, 2005). *E. coli*-expressed chimeric recombinant protein encompassing B-cell epitopes of bmZP1 (132–147 aa), bmZP2 (86–113 aa) and bmZP3 (324–347 aa) generated antibodies in rabbits that inhibited *in vitro* binding of the human sperm to the human zona in the hemizona assay (Sivapurapu *et al.*, 2003). Another elegant approach to design an effective immunocontraceptive vaccine to inhibit fertility may be to employ chimeric recombinant protein comprising of B-cell epitopes of ZP and sperm antigens (Lea *et al.*, 1998; Hardy *et al.*, 2004).

Although various studies have demonstrated the usefulness of ZP glycoprotein-based contraceptive vaccine for the management of animal population, a lot of research and developmental inputs are required to refine these approaches and to understand the immunobiology of each target species (for example, the lessons learned from failure of porcine ZP antigens to inhibit fertility in cats). A crucial issue will be the delivery of the vaccine. These will be unique to each species under study. To make the use of such a vaccine a practical proposition, alternate routes of vaccine delivery, such as oral route in the form of bait or by using darts have to be developed. The use of recombinant organisms for vaccine delivery must address the ethical and safety issues prior to release in the environment. The issue of host specificity must be tested rigorously and it should be built-up at multiple levels in the vaccine. In addition, efforts must be made to develop more potent and safer adjuvants, which will be of immense utility not only for contraceptive vaccines but for vaccines in general. The bottom line will be to ensure that the contraceptive vaccine induces a long-lasting immune response in a high percentage of the recipients, thus reducing the frequency and, hence, the cost of vaccine application.

A comprehensive understanding of the molecular basis of human gamete interaction is required to develop safer and effective contraceptive approaches aiming to intervene at the level of fertilization. Hence, efforts to define B-cell epitopes that are devoid of 'oophoritogenic' T-cell epitopes of ZP glycoproteins must continue. It is imperative that long-term active immunization studies in non-human primates with the identified B-cell epitope-based antigens be undertaken to establish their safety in an unambiguous manner, before these can be considered for human application.

Anti-LHRH/GnRH vaccine

The decapeptide LHRH has some unique characteristics. It is present in males as well as females, hence a vaccine against LHRH is usable in both sexes. Its primary structure is largely conserved in mammals. Thus rodents can be employed as a homologous model for efficacy and safety. Furthermore anti-LHRH approaches can be employed for control of fertility, libido, and sex steroid production in dogs (companion animals), pigs, bulls and heifers (animals raised for meat production). Given that sex steroids promote the growth of accessory reproductive organs such as prostate and breasts, anti-LHRH treatment has therapeutic applications in sex hormone-dependent cancers (Talwar, 1999).

LHRH isomers

In addition to the classical decapeptide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$, which is now represented as GnRH-I, two other isomers of lower vertebrate origin also

exist in humans. GnRH-II, which has three amino acid differences from GnRH-I that was originally isolated from chicken, is also present in human brain (White *et al*., 1998). GnRH-III, which was initially identified in salmon, also exists in humans (Yahalom *et al*., 1999). In humans, the gene coding for GnRH-II is located on the chromosome 20p13, distinct from the gene for GnRH-I located on chromosome 8p21-p11.2. Each isoform has a unique location within the brain, thus suggesting different evolutionarily developmental origins and/or functions (Urbanski *et al*., 1999). The isomers are also expressed in other tissues. The physiological roles of GnRH-II and GnRH-III are not clear in humans and other mammals. The hypothalamic GnRH-1 is clearly the main hormone in the hypothalamic–pituitary–gonadal axis, besides a possible direct role of this decapeptide in extrapituitary organs, such as testis, prostate and placenta. The work reviewed in this paper is on GnRH-I, also referred as LHRH or GnRH.

Semisynthetic vaccines

LHRH by itself is not immunogenic and has to be conjugated to a carrier to mobilize T-helper cells. In view of the absence of a functional group for linkage, an analogue of LHRH was proposed in which glycine at position 6 was replaced by D-lysine creating a spare NH_2 group which could be linked reliably to a carrier such as DT through a spacer (Talwar *et al*., 1992). LHRH-6-DLys-DT was effective in bringing testosterone to castration levels with concomitant atrophy of prostate of rats and monkeys (Jayashankar *et al*., 1989; Giri *et al*., 1991). These findings were confirmed in Austria (Rovan *et al*., 1992) and extended to show that this vaccine inhibited also the growth of Dunning prostate tumour cells in rats (Fuerst *et al*., 1997).

Clinical trials in prostate carcinoma patients

Besides its utility as a contraceptive, the anti-LHRH/GnRH vaccine may also find application in the treatment of prostate cancer. After preclinical toxicology and the Drugs Regulatory and Ethics approvals in India and Austria, this vaccine was taken up for Phase I/Phase II clinical trials in 28 patients of advanced stage carcinoma of prostate (12 patients at the All India Institute of Medical Sciences, New Delhi, India, 12 at the Post Graduate Institute of Medical Education and Research, Chandigarh, India, and 4 at the Urologizche Klinikum, Salzburg, Austria). The vaccine employed alhydrogel, an adjuvant permissible for human use. It was used at 200 μg and 400 μg dose, and three injections were given at monthly intervals. The vaccine was well tolerated by all patients with no side-effect attributable to immunization. A 400 μg dose produced antibody titres >200 pg dose. Patients generating >200 pg of antibodies/ml benefited clinically. Testosterone declined to castration levels. The prostate-specific antigen (PSA) and acid phosphatase declined to low levels. Ultrasonography and serial nephrostograms showed the regression of prostatic mass (Talwar *et al*., 1998).

Two clinical trials with a GnRH-DT vaccine have been carried out at Nottingham, UK by Bishop's group. In the first study (Simms *et al*., 2000), the vaccine was used at two doses,

30 μg and 100 μg, administered three times over 6 weeks in 12 patients with advanced prostate cancer. It was well tolerated and in five patients a significant reduction in serum testosterone and PSA levels was seen. Testosterone declined to castration level in four patients and remained so for 9 months. The response was best in patients with high antibody titres. The dose–response trial was continued in another 12 patients. GnRH-DT vaccine was given intramuscularly at either 3 μg or 15 mg dose per injection thrice. A 3 μg dose was perhaps too low. Two out of six subjects who received 15 μg of GnRH-DT generated significant antibody titres (Parkinson *et al*., 2004).

The above-mentioned clinical studies in India, Austria and UK confirmed the safety of LHRH or GnRH linked to DT vaccine in prostate carcinoma patients. These studies further showed that in patients generating adequate antibodies, testosterone declined to castration levels with concomitant decline of PSA, and there was clinical benefit to the patients.

Alternative carriers

An alternative carrier, namely *Mycobacterium tuberculosis* hsp 70, was linked to GnRH-6-DLys by Delves and Roitt's group employing either Ribi adjuvant or incomplete Freund's adjuvant (Hannesdottir *et al*., 2004). With either adjuvant, all mice made sufficient antibodies to cause atrophy of the uro-genital complex. LHRH-6-DLys was also employed by X.Y.Zeng *et al*. (2002) after conjugation to albumin and was used along with Specol as an adjuvant. It was effective in pigs as an alternative to castration. LHRH-6-DLys, initially proposed by us, has been repeatedly used by others to link various carriers to make bio-effective vaccines. Ovalbumin is another carrier that has been employed for conjugation. Zhang *et al*. (1999) conjugated four or seven LHRH moieties to ovalbumin to obtain recombinant proteins in *E. coli*. LHRH-7 product was more immunogenic than LHRH-4 construct. They both required potent adjuvants for causing sterilization. Stevens *et al*. (2005) fused LHRH to thioredoxin and ovalbumin, each conjugate containing seven LHRH molecules. Both conjugated proteins were as bioeffective as the spaying of heifers, indicating that the immunization against LHRH could be an alternative to castration of male or female animals raised for meat production. Previous studies of the Population Council group (Ladd *et al*., 1990) found that the conjugation of TT at the N-terminal was better than at the C-terminal. Desirability of keeping the LHRH C-terminal free has also been advocated by Ferro *et al*. (2002a). Linkage of TT at the N-terminal in Des 1-GnRH, where glutamic acid at position 1 is replaced by cysteine, gives better conjugate that induces antibodies specific to the classical GnRH-1. Dimerization enhances titres but the monomer conjugate was found to be more effective (Ferro *et al*., 2002b).

Adjuvants and other strategies employed for enhancement of immune response

The conjugation with DT or TT induces antibody response in laboratory animals and in humans with alum as adjuvant, but the linkage with Hsp 70 or with ovalbumin requires stronger adjuvants. W.Zeng *et al*. (2002) proposed the insertion of lipopeptides, dipalmitoyal-*s*-glyceryl cysteine between LHRH

and T-cell epitopes of influenza virus haemagglutinin. This totally synthetic lipopeptide induced anti-LHRH response without any additional adjuvant. A novel retro-inverso GnRH composed of D-amino acids assembled in reverse order (C to N terminus) was found to induce high titres of antibodies reactive with native GnRH without conjugation to a carrier or use of an adjuvant (Fromme *et al.*, 2003). Ferro *et al.* (2004) tested non-ionic surfactant vesicles, aluminium hydroxide, Quil A, polylactide co glycolide acid (PLGA) and Quil A/PLGA combination, with their cysteine-modified LHRH linked to TT. They concluded that PLGA was the most effective adjuvant. Interestingly, Diwan *et al.* (1998) reported that the encapsulation of GnRH-6-DLys-TT in PLGA microspheres induces a bio-effective antibody response within 15 days after a single administration, obliterating the necessity of repeated injections.

Anti-LHRH vaccines for better growth of animals and improvement of quality of meat

Peri- and post-pubertal rams and boars accumulate androgen derivatives, androsterone and skatole in their adipose tissues, which give unpleasant odour to meat. Immunization with an anti-LHRH vaccine reduces testosterone and eliminates the taint, thus improving meat quality (Dunshea *et al.*, 2001). The immunized animals also grow more rapidly than the controls. Oliver *et al.* (2003) carried out a large scale trial in boars and gilts, concluding that Improvac, a vaccine against LHRH and porcine somatotrophin, has synergistic and additive effects on growth of these animals.

Besides the utility of anti-GnRH immunization in animals raised for meat, GnRH vaccine has been employed with good results for control of wild animal populations (Miller *et al.*, 2000; Curtis *et al.*, 2002).

Recombinant vaccines against LHRH/GnRH

Enough data have accumulated to conclude that the vaccines against GnRH-I can be employed in humans and in animals without side-effects. These vaccines have benefit in the treatment of prostate carcinoma patients, are effective in controlling fertility of wild animals and sex steroid hormone production thus regulating estrus and libido of animals raised for meat purposes. Recombinant vaccines would be substantially cheaper to make on an industrial scale than synthetic vaccines.

Hsu *et al.* (2000) conjugated multiple copies of GnRH with receptor-binding domain of *Pseudomonas* exotoxin A. This recombinant protein containing 12 repeats of LHRH along with this carrier generated high antibody titres in rabbits with degeneration of ovaries. They recommend this vaccine for treatment of GnRH-sensitive ovarian cancer. Jinshu *et al.* (2004) have assembled genes for three repeats of GnRH linked through an eight amino acid hinge fragment of human IgG$_1$ to a helper T peptide of measles virus. The DNA coding for a dimer of this complex assembly was fused to the C-terminal (199–326)-encoding sequence of asparaginase. This protein was expressed in *E. coli* and generated anti-LHRH response. Talwar *et al.* (2004) reported the ability of a multimer recombinant anti-LHRH vaccine to cause decline of testosterone to castration level and atrophy of prostate of rats. In the design of

this vaccine, DT/TT used as carriers in the previous semi-synthetic vaccines were replaced by four or five T non-B-cell peptides interspersed in four or five LHRH units. This was done to avoid carrier-induced epitope suppression brought by DT/TT carrier conjugates (Sad *et al.*, 1991), and also to communicate through an array of these T-cell determinants with MHC across the spectrum in a polygenetic population. The genes were assembled, cloned and expressed at high level (15% of total cellular protein) in *E. coli* (Gupta *et al.*, 2004). Employing a buffer at pH 3, it was possible to extract the protein from inclusion bodies employing low concentrations of chaotropic reagents (2 mol/l urea instead of 8 mol/l). The protein was purified and refolded to native immunoconformation (Raina *et al.*, 2004).

Finstad *et al.* (2004) have also employed the strategy of linking LHRH to four promiscuous T-helper sequences and in some cases linked also to 'adjuvanting' peptide from *Yersinia* invasin. Their vaccine is at this stage synthetic and not recombinant. The antibodies generated are directed at LHRH. The bulk of antibodies formed against the carriers (DT/TT) are avoided, and as expected no antibodies are formed against T non-B cell peptides used as carriers.

Anti-HCG vaccine

β-HCG and HSD-TT/DT vaccine

A vaccine against HCG is the first and only birth control vaccine to go through Phase II efficacy trials successfully and it has been shown to protect sexually active women from becoming pregnant (Talwar *et al.*, 1994). The vaccine was highly effective and only one pregnancy occurred in 1224 cycles so long as the antibody titers remained >50 ng/ml. The vaccine was fully reversible and women conceived readily when the antibody titres declined to <35 ng/ml. The uniqueness of this vaccine is that it is directed against a molecule which is made in detectable amounts only in pregnancy in healthy women. The vaccine is devoid of side-effects, as observed in >200 women during Phase I and Phase II trials. Women keep ovulating normally, make their own sex hormones and the menstrual cycles are regular. No irregularity of bleeding in terms of spotting, amenorrhoea or extra bleeding occurs (Talwar *et al.*, 1997). The fact that luteal phase did not lengthen in vaccinated women provided confirmation of the previous work in marmosets (Hearn *et al.*, 1988) on anti-HCG antibodies preventing implantation of the embryo onto the endometrium. Interception is therefore before the onset of pregnancy.

While the proof of concept is largely given by these studies, the shortcoming of the vaccine was that it generated above protective threshold titres in only 60–80% of women. This degree of efficacy is highly satisfactory for vaccines against infectious diseases but a birth control vaccine has to be effective in >90–95% of recipients in order to be acceptable. Further work on product development is required. It would require more potent adjuvants, which are available today from the pharmaceutical industry, instead of the alum employed in the test vaccine. At this time, there is also a need constantly to monitor the vaccinated individuals for the presence/absence of sufficient antibody titres that can block conception.

T non-B-cell peptides as carriers

Although both DT and TT used as carriers evoke good antibody response, the repeat immunization with β-HCG-TT vaccine causes a carrier induced epitope suppression in some women. To overcome this, an alternative carrier has to be used (Gaur *et al.*, 1990). It would be logical to replace DT/TT by a set of promiscuous T non-B-cell peptides, which could communicate with major histocompatibiliy complex (MHC) of a polygenetic dispersed population. Pilot studies have shown that the conjugation of β-HCG to a cocktail of peptides not only enhances the quantum of immune response but also assures antibody response in mice of different genetic background. The peptides employed were from measles virus, human immunodeficiency virus (HIV)-1 reverse trancriptase and influenza virus haemagglutinin. While conjugation with each peptide improved antibody response against HCG in some genetic strains of mice, use of combination of these peptide conjugates generated anti-HCG response in all mice of different genetic background and the titres were higher than those achieved with individual peptides or with β-HCG conjugated to TT (Gupta *et al.*, 2001).

Live vector engineered β-HCG vaccine

Srinivasan *et al.* (1995) observed that β-HCG expressed along with a 48 amino acid membrane anchor in vaccinia generated high titre anti-HCG antibodies of long duration in rodents. Although the vaccinia used was an attenuated virus, its flare-up in immunocompromised subjects cannot be ruled out. Thus a construct in fowl pox virus was made which expresses the engineered protein but does not replicate in humans (Gupta *et al.*,1998).

β-HCG-CTP vaccine

The other vaccine against HCG was developed by Vernon Stevens with support from the WHO Task Force on Vaccines for Fertility Regulation. It is based on a portion (the carboxy-terminal peptide or CTP) of the β subunit of the hormone (β-HCG-CTP) linked to DT. The antibodies generated by the CTP vaccine are totally devoid of cross-reactivity with human LH. However, the CTP antibodies react with the somatostatin-producing cells of the pancreas, whereas the antibodies generated by the β-HCG and HSD vaccines being conformational did not exhibit such tissue reactivity as tested by Rose *et al.* (1988). The Phase I clinical trials were conducted with the CTP vaccine in 20 women in Australia (Jones *et al.*,1988). The antibody titres were low and had low affinity for HCG ($K_a \approx 10^8$ L/mol) compared to β-HCG or HSD-induced antibodies ($K_a \approx 10^{10}$ L/mol) (Singh *et al.*, 1989). The CTP vaccine underwent a Phase II trial in Sweden but this was abandoned due to unacceptable reactions caused in vaccinated women.

Rock *et al.* (1996) engineered a fusion protein consisting of *E. coli* heat-labile enterotoxin subunit B (LTB) genetically linked at its C terminus via a nine amino acid linker to the 37 amino acid carboxyl terminal peptide (CTP) of the HCG β chain. LTB-CTP fusion protein was stably expressed in bacteria and formed pentamers. Purified LTB-CTP protein induced HCG-specific antibodies in mice without additional adjuvants. No further publication on this vaccine is available on efficacy.

Another approach for enhancement of immunogenicity of peptide-based contraceptive vaccine is proposed by Kvirkvelia *et al.* (2003) by using hepatitis β-core antigen particle as presentation scaffold.

Generation of recombinant β-HCG mutants devoid of human LH cross-reaction

In order to eliminate or reduce cross-reactivity with human LH, site-directed mutants of the HCG β-chain have been generated. These mutants were screened for reactivity with a panel of monoclonal antibodies, both reactive and non-cross-reactive with human LH and those which recognize one or more conformational epitopes specific to native β-HCG. A number of candidates were identified in which the mutants had changes at amino acids such as Arg-68, Lys20, Glu-21 and Gly-22 (Roitt and Delves, 1997). β-HCG with single amino acid substitution for Arg-68 by glutamine, generated β-HCG-reactive but non-human LH cross-reactive antibodies (Chiesa *et al.*, 2001; Porakishvili *et al.*, 2002). This group proposed such mutants for use, either as DNA or protein vaccines for fertility control (Delves and Roitt, 2002). However, this group as well as others have noted that DNA vaccination creates memory, but gives poor antibody response (Laylor *et al.*, 1999).

Passive use of preformed antibodies

An alternative way to assure efficacy in nearly 100% of cases would be to administer an adequate quantity of preformed bio-effective antibodies for intervention. A mouse monoclonal having a high affinity ($K_a = 3 \times 10^{10}$ L/mol) and high specificity for HCG with no cross-reaction with human FSH and human TSH and <5% cross-reaction with human LH was developed. This antibody has been converted into a chimeric recombinant antibody (cPIPP) in which the variable part of the mouse monoclonal has been fused with the human IgG$_1$ and human κ chain (Kathuria *et al.*, 2002a). It has been expressed in plants at a yield of 20 mg/kg of fresh leaves (Kathuria *et al.*, 2002b). The antibody retains the affinity and high specificity of mouse monoclonal, but consists mostly of the part corresponding to a human antibody. It is bio-effective and prevents HCG-induced increase of uterine weight in mice (Talwar, 2003). More interestingly, it has been effective in neutralizing HCG action in a human-derived test system. It prevents the fusion of human placental cytotrophoblasts to syncytiotrophoblasts, which takes place under the influence of endogenous HCG and inhibits the secretion of progesterone by these cells (Dhar *et al.*, 2004). Besides its use as a vacation (4 weeks) contraceptive, the passive immunization with antibodies may find useful applications in imaging, selective delivery of radiations/drugs, and immunotherapy of advanced stage drug-resistant (relapse) cancers.

Ectopic expression of HCG in various cancers

Traditionally it was believed that HCG is made only in pregnancy, hence its presence in serum or urine continues to be a reliable diagnostic test for pregnancy. Being a trophoblast product, HCG is present in measurable amounts in malignant trpophoblastic tumours (Hameed *et al.*, 1999; Sahraoui *et al.*,

2001; Tinkle *et al.*, 2001; Weiss *et al.*, 2001). In recent years, several studies have reported the expression of α, β or both subunits of HCG in a variety of cancers (Table III). The ectopic synthesis of HCG is not limited to a given type of cancer. It is further reported that such cancers have poor prognosis and adverse survival rate (Syrigos *et al.*, 1998). It can be hypothesized that with ultra-dedifferentiation, the tumour cells assume embryonic character, hence they start synthesizing this oncofetal protein. Invariably, the cancers at this stage are drug resistant, and the tumour is spread over various organs by metastasis. Thus humanized antibodies specifically reacting with tumour cells and with no other tissue of non-pregnant female can be of special interest for imaging and selective delivery of drugs and radiations to tumour cells.

It was observed that the cPIPP, the chimeric antibody described above, binds onto the membrane of MOLT-4, a T-lymphoblastic leukaemia cell line (American Type Culture Collection, ATCC) from a relapse case. The antibody exhibits no binding with peripheral blood mononuclear cells of healthy normal individuals. This antibody specifically binds to JEG tumours in nude mice, as compared to the control antibody. Anti-HCG antibodies did not bind to non-HCG synthesizing SP2/O cell line (Kaur, 1989; Talwar, 2003). The role of HCG or its subunits with respect to the biology of the tumour cells is not fully clear. It was reported that Chago lung cancer cells make α-HCG which acts as a growth promoter for these cells. Antisense RNA or antibodies that neutralize α-HCG inhibit the growth of these cells *in vitro*. Antibodies given along with tumour cells prevented the growth of the tumour in nude mice in a dose-dependent manner (Kumar *et al.*,1992). If the antibodies are administered at a stage when the tumour is already established in the nude mice, they cause necrosis of the tumour. These studies point to an autocrine growth-promoting role of HCG or its subunits on such tumours.

Antibodies directed to membrane-localized epitopes could lyse the cells in the presence of complement. This was found to be the case with a monoclonal MoAb 730 developed against androgen-independent DU-145 cells (Talwar *et al.*, 2001). Antibodies could also exercise antibody-dependent cytotoxicity (ADCC) on tumour cells. These are areas where more work is needed and there is a possibility that chimeric antibodies against HCG or its subunits may herald new therapeutic approaches for advanced stage cancers, for which no effective therapy exists at present.

Table III. Ectopic expression of HCG or its α, β subunits in various cancers

Cancer	Reference
Lung cancer	Szturmowicz *et al.*, 1999; Ikura *et al.*, 2000
Urothelial cancer	Iles and Chard, 1989
Colon adenocarcinoma	Kiran *et al.*, 2001; Lundin *et al.*, 2001
Pancreatic carcinoma	Taylor *et al.*, 1990; Alfathan *et al.*, 1992
Liver malignancies	Hoermann *et al.*, 1992
Neuroendocrine tumours	Bidart *et al.*, 1997
Bladder retroperitoneal teratoma	Okamoto *et al.*, 2001
Pancreatic carcinoma	Louhimo *et al.*, 2001; Okamoto *et al.*, 2001
Cervix carcinoma	Hameed *et al.*, 1999

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