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Prospects of riboflavin carrier protein (RCP) as an antifertility vaccine in male and female mammals

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Riboflavin carrier protein (RCP) is obligatorily involved in yolk deposition of the vitamin, riboflavin, in the developing oocyte of the hen. The production of this protein is inducible by oestrogen. It is evolutionarily conserved in terms of its physicochemical, immunological and functional characteristics. It is the prime mediator of vitamin supply to the developing fetus in mammals, including primates. Passive immunoneutralization of the protein terminates pregnancy in rats. Active immunization of rats and bonnet monkeys with avian RCP prevents pregnancy without causing any adverse physiological effects of the mother in terms of her vitamin status, reproductive cycles or reproductive–endocrine profile. Denatured, linearized RCP is more effective in eliciting neutralizing antibodies capable of interfering with embryonic viability either before or during peri-implantation stages. Two defined stretches of sequential epitopes, one located at the *N*-terminus and the other at the *C*-terminus of the protein have been identified. Active immunization with either of these epitopes conjugated with diphtheria toxoid curtails pregnancy in rats and monkeys. Immunohistochemical localization of RCP on ovulated oocytes and early embryos shows that the antibodies cause degeneration only of early embryos. RCP is produced intra-testicularly and becomes localized on acrosomal surface of mammalian spermatozoa. Active immunization of male rats and monkeys with denatured RCP markedly reduces fertility by impairing the fertilizing potential of spermatozoa. These findings suggest that RCP, or its defined fragments, could be a novel, first generation vaccine for regulating fertility in both the sexes.

Key words: antifertility vaccine/female contraception/male contraception/riboflavin carrier protein

Introduction

The developing mammalian embryo is continuously nourished by the mother. It accumulates many essential nutrients, including water-soluble vitamins, against concentration gradients (Miller *et al.*, 1976). Riboflavin, one of the vitamins indispensable for embryonic growth, is transported across the feto–placental membranes by a specific protein called riboflavin carrier protein (RCP) (Adiga *et al.*, 1986). Interference with maternal RCP by passive or active immunization with heterologous

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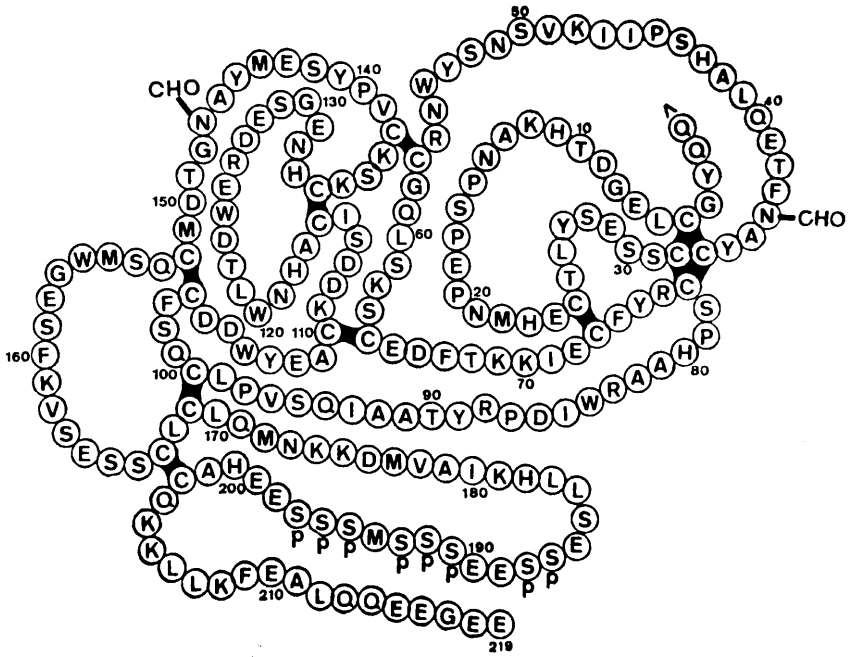

Figure 1. The primary structure (single letter code) of the chicken egg-white riboflavin carrier protein. <Q, *N*-terminal pyroglutamic acid; P = phosphate residue; CHO = asparagine-linked oligosaccharide (Reproduced from Hamazume *et al.*, (1984) with the permission of the authors).

(chicken) RCP, results in early embryonic loss in rats (Murty and Adiga, 1982; Adiga and Murty, 1983) and bonnet monkeys (Seshagiri and Adiga, 1987; Adiga *et al.*, 1991) without disrupting subsequent reproductive cycles, the reproductive–endocrine profile or the vitamin status of the mother. These findings highlight the important role of this evolutionarily conserved protein as a prime mediator of flavin transport to growing mammalian embryos.

RCP is produced under oestrogenic influence by somatic cells in the rat testis and is localized on the acrosomal surface of mature spermatozoa. Active immunization of male rats and bonnet monkeys with RCP results in antibodies coating the sperm surface and also significantly impairs male fertility (Bhat *et al.*, 1995; Subramanian and Adiga, 1996a,b; Sridhar *et al.*, 1996).

This article reviews research carried out in our laboratories aimed at assessing the feasibility of RCP as an antifertility vaccine for both men and women.

RCP in the chicken oocyte

RCP was first identified and characterized in the chicken egg white and yolk. It is a phosphoglycoprotein (molecular weight 37 000) exhibiting high-affinity (Ka 10^{7}–^{108}/M) to free riboflavin (White and Merril, 1988; Adiga, *et al.*, 1988, 1994). This oestrogen-inducible vitamin carrier is present in the circulation of egg-laying birds but not in immature pullets or adult cocks (Murthy and Adiga, 1978). Following oestrogen induction and hepatic secretion, RCP forms a tight complex with circulating riboflavin which is then deposited in the yolk of the developing oocyte. Additional RCP, secreted together with other egg white proteins by the oviductal magnum, is incorporated around the oocyte (Blum, 1966; White and Merrill, 1988). RCP in the yolk and albumin compartments of the egg are products of the same gene. Yolk RCP, however, lacks 11–13 amino acids at the carboxy terminus as a result of specific proteolytic processing during oocyte deposition (Hamazume *et al.*, 1984). The carbohydrate composition of yolk RCP is identical to serum RCP, which in turn, differs from that of egg white RCP. Phosphorylation of RCP is confined to a short stretch of serine residues near the *C*-terminus (Figure 1).

Yolk deposition of vitamin-saturated RCP meets the nutritional requirement of the growing embryo. The essential need of RCP for the survival of the developing avian embryo is exemplified by the finding of a mutant strain of chicken which exhibits riboflavinuria (Clagett, 1971). The gene coding for RCP is non-functional in these birds due to aberrant splicing events (MacLachlan *et al.*, 1993) resulting in a failure for riboflavin deposition and non-hatching of the egg. Affected progeny can however be rescued by injecting the vitamin directly into the fertilized egg. Growth, riboflavin metabolism, sexual maturity and egg laying potential of birds homozygous for the defective RCP gene are comparable with normal controls (White

et al., 1992). Obviously the plasma membrane of the oocyte constitutes a barrier to the entry of free, unbound riboflavin. RCP is a reproduction-specific protein with the essential physiological role of depositing riboflavin into the oocyte.

Some information is available regarding the functional domains of chicken RCP with reference to riboflavin binding at the hydrophobic pocket in the protein's interior and putative receptor recognition sites on the surface. Chemical modification of the oligosaccharide chains significantly reduces its uptake at the oocyte plasma membrane (Miller *et al.*, 1981a,b) despite the fact that vitamin-binding characteristics remain undisturbed. The importance of the phosphopeptide region of RCP, in terms of its deposition of the vitamin in the oocyte, is illustrated by the observation that dephosphorylation even at a single site among eight serine residues curtails vitamin uptake by 60% (Miller *et al.*, 1982). The bulk of evidence favours the view that RCP transport through oocyte plasma membrane may be a complex process involving multiple recognition sites on the protein surface. Recent attempts to identify RCP receptors on chicken oocyte membrane have revealed that ligand binding to these receptors was Ca^{2+}-dependent and inhibited by vitellogenin. Ligand-blotting showed multiple receptors of molecular size 360, 260 and 130 kDa located on the avian oocyte membrane. Biochemical evidence suggest that these receptor entities belong to the family of low-density lipoprotein receptor related proteins that mediate oocyte uptake of RCP. The dominant role of the phosphopeptide domain of RCP in receptor recognition becomes clear from ligand blotting experiments where the purified phosphopeptide (residues 182–204) drastically inhibits RCP binding (Sarkar *et al.*, 1996).

Evolutionary conservation

Rodents

The presence of RCP homologue in pregnant rat serum suggests its putative role in mammalian reproduction. Like its counterpart in the hen, the rodent protein is oestrogen-dependent (Murty and Adiga, 1982). When preformed, specific rabbit antibodies to chicken RCP were administered to pregnant rats on day 11 of gestation, the pregnancy was terminated and 100% fetal resorption occurred (Adiga *et al.*, 1986). Embryonic wastage following such passive immunization is due to a drastic reduction in riboflavin transport to the fetus (Adiga and Murty, 1982). Diminished flavin supply causes severe reduction in concentrations of flavin co-enzymes, particularly marked depletion in flavin adenine dinucleotide (FAD) content below the minimum threshold to sustain embryonic growth. Histological examination of the affected fetoplacental unit revealed a separation of the placental membrane from the decidua, leukocyte infiltration into the embryonic liver and trophoblast degeneration (Adiga *et al.*, 1988). Female rats of proven fertility, actively immunized with chicken RCP and mated with fertile males, showed bloated uterine horns at laparotomy on day 8 post-mating which were similar to those seen during early or pseudo-pregnancy indicating early embryonic loss. None of the actively immunized animals delivered pups and this phenomenon lasted as long as antibody titres remained high. Pregnancies were carried to term when antibody titres dropped demonstrating the reversibility of the immunointerference. Homologous RCP is not required for maternal well-being because no adverse reaction could be detected in immunized rats (Adiga and Murty, 1983). Presumably RCP has a specific role of channeling riboflavin to the developing embryo.

Bonnet monkeys (Macaca radiata)

A protein similar to chicken RCP is also present in pregnant monkey serum (Visweswariah and Adiga, 1987). The purified primate protein exhibits physicochemical properties identical to chicken RCP (Visweswariah and Adiga, 1988). Circulating concentrations of monkey RCP is also modulated by oestrogen. Mid-cycle and early pregnancy rises in oestradiol concentrations is accompanied by an increase in plasma values of RCP (Visweswariah and Adiga, 1988). Pharmacological doses of oestrogen raise blood concentrations of RCP in immature male or female monkeys; the magnitude of response in males is less pronounced than in females (Visweswariah and Adiga, 1988).

Proven fertile monkeys actively immunized with purified, homogenous chicken RCP produced antibodies that cross-react with [^{125}I]-labelled purified monkey RCP (Adiga *et al.*, 1991). Active immunization with heterologous RCP does not change the characteristics of the menstrual cycle, the ovarian-endocrine function or the riboflavin status. Of the 14 established pregnancies, 50% failed after high-titre antibodies were elicited. However, the duration of pregnancy varied from 15 to 60 days (Seshagiri and Adiga, 1987). These data indicate the crucial role played by RCP in primate reproduction. However, the prospect of using chicken RCP as an antipregnancy vaccine in monkeys at this stage was not promising.

Structural modification of RCP for eliciting bioeffective antibodies

The magnitude of immune response to RCP, as assessed by total antibody titres, cannot be correlated with pregnancy

outcome. This indicates that the type and content of specific bioneutralizing antibody population, rather than the total antibody repertoire, dictates the efficacy of immunointerference with RCP function *in vivo*.

Sodium dodecyl sulphate (SDS)-denatured RCP is more efficacious as the immunogen than native RCP in terminating pregnancy in rats and monkeys (Adiga *et al.*, 1988), although the magnitude of immune response to the denatured immunogen is relatively less when compared with native RCP (Karande and Adiga, 1991). This finding clearly indicates the relative importance of the types of epitopic conformations (assembled or sequential) on chicken RCP surface in eliciting neutralizing antibodies. SDS-denatured RCP elicited antibodies directed against sequential epitopes preferentially, as measured by their ability to bind linearized RCP (by disulphide reduction and carboxy-methylation; RCM). SDS treatment of RCP enhances the α-helical content as revealed by circular dichroism (CD) spectral measurement, indicating the importance of antibodies against α-helical sequential epitopes in eliciting neutralizing antibodies. A retrospective comparison of the nature and titres of antibodies in monkeys immunized with chicken RCP and which carried their pregnancy to term clearly supports the above hypothesis (Adiga *et al.*, 1991). It may be mentioned that while monkeys that did not carry their pregnancy to term as well as those that did, had comparable values of total antibody titres, monkeys that failed to take their pregnancy to term exhibited relatively higher titres of antibodies directed to sequential epitopes when assessed by their binding to unfolded RCP as the probing antigen. The logical assumption is that antibodies to chicken RCP directed to folded conformation of the protein are redundant in terms of immunoneutralization. Consequently, completely unfolded SDS-treated, reduced and carboxy methylated RCP (SDS–RCM–RCP) is the immunogen of choice for active immunization to elicit higher titres of sequence-specific neutralizing antibodies for more efficient pregnancy curtailment in monkeys following active immunization.

Effects of active immunization with SDS–RCM–RCP on reproductive performance of female Bonnet monkeys

Monkeys of proven fertility and exhibiting regular menstrual cyclicity were administered subcutaneously with SDS-treated RCM–RCP emulsified in alhydrogel (Superfos Biosector a/s, Vedback, Denmark) and periodically boosted to generate reasonable titres of antibodies which recognized both native and RCM–RCP. None of the monkeys became pregnant when mated with fertile males on days 10–16 of their menstrual cycle (Table I). After primary immunization schedule of 3 monthly doses, it was necessary to boost immune response at 4 monthly intervals to sustain high titre antibodies. In some of the immunized animals, the duration of a few menstrual cycles was lengthened by 7–8 days and concomitantly circulating concentrations of progesterone was marginally elevated on day 26 of the cycle, which according to the normal pattern observed in our colony, is usually lower in monkeys with cycle length of 28 ± 2 days. It is possible that these animals might have conceived, but experienced early embryonic loss, giving the appearance of 'near normal' cyclicity (Adiga *et al.*, 1991). These experiments are continuing with a view to ascertain the reversibility of this approach of immunointerference with pregnancy.

Potential stage of immunointerference with pregnancy

The above observations with Bonnet monkeys reveal that interference with pregnancy may operate earlier to the establishment of the placental barrier, perhaps at the peri-implantation stage itself. Earlier studies on rats actively immunized with chicken RCP often showed decidual chambers representing implantation sites at laparotomy on days 7–8 after mating (Murty and Adiga, 1982). Histologically the tissue was pycnotic and showed signs of early fetal resorption. A more detailed examination of this phenomenon after active immunization with SDS-treated RCM–RCP revealed that >90% of the immunized animals had only bloated uterine horns with no evidence for decidual chambers. Presumably the absence was due either to interference with fertilization or early preimplantation embryonic loss. These findings, together with our observation that Pontamine dye reaction as an early signal of embryonic implantation (Psychoyos, 1973) was negative in these immunized animals, suggest that embryonic loss had occurred at the peri-implantation stage (Rao *et al.*, 1995). Earlier findings that RCP can be visualized immunohistochemically on ovulated oocytes as well as early cleaving embryos (including blastocyst) in mated female rats are in line with the premise that oocyte as well as early preimplantation embryo could be a target for antibody-mediated cytotoxicity and degenerative processes. Of relevance in this context is the observation that marmoset embryos exposed to antibodies against β human chorionic gonadotrophin (HCG) fail to implant (Hearn *et al.*, 1988). In-vitro experiments on embryonic development in culture should help to further clarify early

stage(s) at which the RCP antibody interferes with early pregnancy.

Table I. Protection from pregnancy in Bonnet monkeys immunized with sodium dodecyl sulphate-treated linearized riboflavin carrier protein (RCP). Each experimental animal was s.c. administered 300 μg of the antigen emulsified in alugel + SPLPS followed by two boosters of 200 μg each at monthly intervals. Additional boosters were given 4 months later. Animals were mated during day 10–16 of that cycle.

Monkey no.	Total no. of protected ovulatory cycles of individual monkeys[a]	Pregnancy
909	21	Nil
956	30	Nil
945	14	Nil
946	15	Nil
920	13	Nil
182	13	Nil
922	7	Nil
931	8	Nil
Total	121	

[a]Normal ovulation was assessed when the oestradiol concentration was >200 pg/ml (days 8–10) and progesterone concentration >2.5 ng/ml on days 18–20.

Table II. Protection from pregnancy in Bonnet monkeys immunized with CTP–DT conjugate in FCA/FIA. Each experimental animal was s.c. administered 500 μg of the CTP–DT conjugate emulsified in FCA followed by two boosters of 200 μg each in FIA. Additional boosters of 200 μg of the antigen were given at 4 monthly intervals.

Monkey no.	Total no. of protected ovulatory cycles of individual monkeys[a]	Pregnancy
943	24	Nil
944	18	Nil
913	22	Nil
908	10	Nil
911	4	+ (5th Cycle)
Total	78	

[a]Normal ovulation was assessed when the oestradiol concentration was >200 pg/ml (days 8–10) and progesterone concentration was > .5 ng/ml on days 18–20.
CTP = a 21 amino acid peptide corresponding to the *C*-terminal fragment of RCP; DT = diphtheria toxoid; FCA/FIA = Freund's Complete Adjuvant (for first injection)/Freund's Incomplete Adjuvant (for subsequent injections).

Evaluation of *C*-terminal peptide of RCP as an immunogen

Bioneutralizing characteristics of polyclonal antibodies to RCM–RCP prompted investigations on identification of sequential epitopes in the primary structure of RCP that elicit neutralizing antibodies. A panel of seven monoclonal antibodies (mAbs) to chicken RCP showed that all of them recognize purified rat, bovine, monkey and human RCPs. This supports the view that the surface topology of the protein is conserved through evolution (Adiga *et al.*, 1988). One of the mAbs, 6B2C12, recognizes a sequential epitope in both the native and unfolded chicken egg white RCP but not its yolk counterpart (Adiga *et al.*, 1988). This implies that this epitope comprises a part of *C*-terminal peptidyl sequence (residues 207–219) which is missing in yolk RCP. This epitopic sequence is functionally important for eliciting neutralizing antibody since ascites fluid of mAb 6B2C12 administered to pregnant mice terminates pregnancy (Karande *et al.*, 1991). Epitope mapping by Pepscan method employing overlapping synthetic peptides of defined sequence corresponding to *C*-terminus (residues 200–219) revealed its core sequence (residues 197–212) to be ^{203}QKKLLKFEAL^{212}. Antibodies directed against a synthetic peptide comprising this epitope could neutralize the maternal RCP *in vivo*. A 21 amino acid peptide (CTP) corresponding to the *C*-terminal fragment of RCP, Y^{200}HACQKKLLKFEALQQEEGEE^{219} (with a propensity for α-helical conformation in presence of helix-inducing agents), was synthesized and was shown to interact with mAb 6B2C12. An additional tyrosine residue at *N*-terminus served as an aromatic reporter as well as in conjugation to diphtheria toxoid (DT) for T cell help.

Both the synthetic peptide and its DT conjugate bound polyclonal RCP antisera as well as mAb 6B2C12 (Adiga *et al.*, 1993). The CTP–DT conjugate is immunogenic in rabbits, rodents and monkeys eliciting antibodies recognizing CTP, RCP and RCM–RCP. In terms of reactivity *in vivo*, administration of anti-CTP antibodies, as purified immunoglubulin (Ig)G, to Swiss mice on five consecutive days starting from day 9 of pregnancy, resulted in embryonic resorption in all the animals. This is consistent with efficient immunoabrogation of RCP *in vivo* by the antipeptide antibodies (Koshy *et al.*, 1996).

A group of five proven fertile monkeys with regular menstrual cycles were immunized with CTP–DT conjugate in Freund's Complete Adjuvant (for first injection)/Freund's Incomplete Adjuvant (for subsequent injections) (FCA/FIA) as adjuvant. After three monthly injections, when a good immune response to the peptide occurred, the monkeys were mated with fertile males between days 10–16 of the menstrual cycle and blood

levels of oestrogen and progesterone were monitored. Only one monkey became pregnant during the fifth mated cycle while the others did not (Table II). Immunization *per se* did not elicit any discernible adverse effect on the general health of the animals. These observations support the immunocontraceptive potential of CTP as a synthetic peptide vaccine.

Table III. Protection from pregnancy in Bonnet monkeys immunized with NTP–DT conjugate in FCA/FIA. Each experimental animal was s.c. administered 500 μg of NTP–DT conjugate emulsified in FCA followed by two boosters of 200 μg each in FIA. Additional boosters of 200 μg of the antigen were given at 4 monthly intervals.

Monkey no.	Total no. of protected ovulatory cycles of individual monkeys[a]	Pregnancy
271	9	Nil
910	8	Nil
4008	8	Nil
4013	7	Nil
Total	32	

[a]Normal ovulation was assessed when the oestradiol concentration was >200 pg/ml (days 8–10) and progesterone concentration was >2.5 ng/ml on days 18–20.
CTP = a 21 amino acid peptide corresponding to the *C*-terminal fragment of RCP; DT = diphtheria toxoid; FCA/FIA = Freund's Complete Adjuvant (for first injection)/Freund's Incomplete Adjuvant (for subsequent injections).

Evaluation of an *N*-terminal peptide (NTP) of RCP as the immunogen

In a search for additional epitopic structures capable of eliciting neutralizing antibodies to RCP, an amino acid sequence (^{4}GCLEGDTHKANPSPEPNMHEC^{24}) was recognized at the *N*-terminus as a potential antigenic segment on theoretical grounds based on algorithms to predict hydrophilicity profile, flexibility, antigenic index and secondary structure. A 21-residue *N*-terminal peptide (NTP), ^{1}GALEGDTHKANPSPEPNMHEY^{21}, corresponding to the nested sequence 4–24 of chicken RCP (Figure 1) wherein cystine residues at positions 5 and 24 were respectively altered to Ala and Tyr, was synthesized and coupled to DT for immunization in rabbit. Epitope mapping by Pepscan method using rabbit antipeptide immune serum as well as polyclonal antisera raised to native or RCM–RCP revealed three core sequences, i.e. ^{2}ALEGDT^{7}, ^{11}NPSPE^{15} and ^{16}PNMHE^{20}. These correspond well to conformationally defined elements of structure (Beena, 1995). The antipeptide antibodies could bind the native- and RCM–RCP (as well as NTP) in enzyme-linked immunosorbent assays and immunoblots showing that corresponding peptidyl segment is surface-exposed in the native folded structure. The propensity of these NTP antibodies (administered as IgG) to interact with RCP *in vivo* was evident when administered to proven pregnant mice for three consecutive days from day 8 of pregnancy. Such a treatment resulted in 100% early embryonic resorption whereas the control animals receiving similar dosage of non-immune IgG carried their pregnancies to term to deliver normal pups. These observations clearly prove the bioefficacy of the antibodies to RCP–NTP to interfere with pregnancy progression in rodents.

Extension of these studies to female Bonnet monkeys have shown that NTP–DT conjugate can indeed elicit antibodies specifically recognizing the NTP as well as the native and RCM–RCP with good titre without interfering with their menstrual cyclicity, hormonal profiles and general well being. In preliminary studies to test the efficacy of active immunization with NTP–DT conjugate, a group of four monkeys of established fertility and regular cyclicity were mated with fertile males during days 10–16 of ovulatory cycle over a period of 2 years. None of them so far have become pregnant after exposure to males during 7–9 fertile cycles (total protected cycles 32) (Table III). These encouraging observations are being further extended for long-term efficacy and reversibility of immunointerruption of pregnancy.

RCP in male reproduction

The possibility that RCP is elaborated intra-testicularly was studied because of its possible role in facilitating vitamin transport for germ cell proliferation and differentiation. This premise is based on the following rationale: (i) spermatogenesis in adult mammalian testis involves active and continuous cellular proliferation and differentiation despite the constraint offered by an effective blood testis barrier due to tight junctions of the Sertoli cells which isolate the germ cells from extratubular environment; consequently, all nutrients and growth factors are either supplied by or transported across the Sertoli cells (Bardin *et al.*, 1988); (ii) several carrier-mediated nutrient transport systems operate through this physiological barrier to sustain uninterrupted germ cell development (Bardin *et al.*, 1988; Skinner, 1991); (iii) evidence is unequivocal for the production of oestrogen by both the Sertoli and Leydig cells and its importance in testicular function (Dufau, 1985). Initially, we succeeded in identifying RCP immunohistochemically in testicular sections on both the Leydig and Sertoli cells as well as on isolated pachytene spermatocytes, round and elongated spermatids of the adult rat. The protein could be clearly visualized on the acrosomal region of the mature

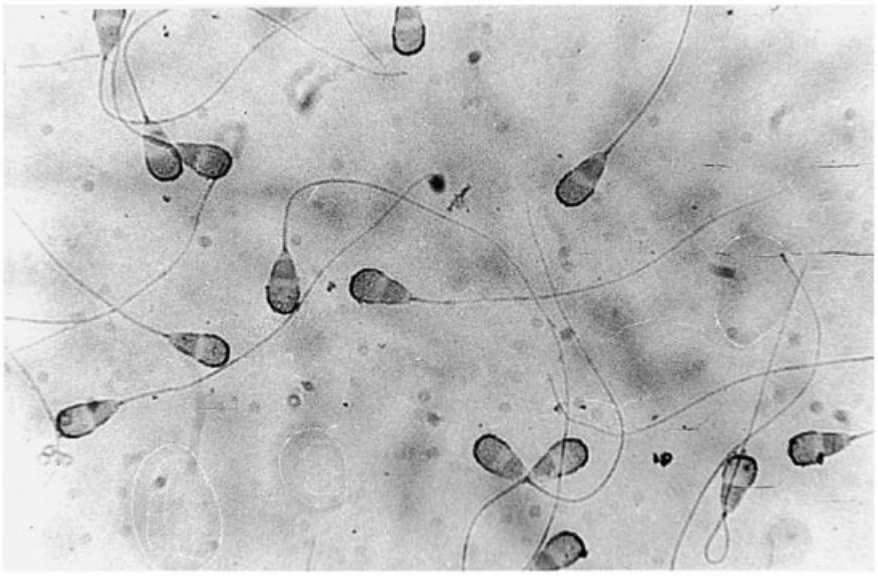

Figure 2. Immunohistochemical localization of riboflavin carrier protein (RCP) on bovine spermatozoa. Ejaculated spermatozoa separated from seminal plasma were thoroughly washed with phosphate-buffered saline containing 0.3% bovine serum albumin smeared on microscopic glass slides, air-dried, fixed and stained for RCP by indirect immunoperoxidase staining technique (original magnification ×1000) (Bhat *et al.*, 1995).

spermatozoa from rats, cattle, monkeys and humans (Bhat *et al.*, 1995) (Figure 2). More recent investigations have shown that the purified Sertoli cells from the immature rats and the Leydig cells from the adult rats elaborate RCP in culture in response to gonadotrophic stimulations through enhanced intracellular production of oestrogen (Subramanian and Adiga, 1996a,b). While the details of involvement of RCP in the transport to and sequestration of riboflavin by the germinal cells during proliferation and differentiation in the intratubular environment await further study, it is conceivable that the vitamin transport is analogous to iron transport involving testicular transferrin (Griswold, 1988).

We subsequently found that immunostainable RCP in the acrosomal cap of the washed epididymal spermatozoa in the rat and electro-ejaculated monkey spermatozoa is surface-exposed and is accessible to antibody binding as seen by direct staining for sperm bound antibody as well as by flow cytometric analysis of paraformaldehyde-fixed spermatozoa using secondary antibody fluorescein isothiocyanate (FITC) conjugate. This prompted us to investigate changes in sperm characteristics in the presence of exogenously added RCP antibody (IgG). The antibody had a profound effect on the sperm motility *in vitro* in culture; in addition to their inability to attain hypermotility *in vitro* (indicative of capacitation); they were progressively immobilized with time, which was further aggravated in presence of guinea pig serum as a source of complement (Sridhar *et al.*, 1996). As assessed by zona-denuded hamster egg penetration test (Yanagimachi *et al.*, 1976), such debilitated spermatozoa exhibited markedly impeded (>70%) capacity to penetrate the ovum investments.

Encouraged by these in-vitro findings, we examined the effect of active immunization of male rats with RCM–RCP on their sperm characteristics *in vitro* and fertility on mating with non-immunized fertile females (Sridhar *et al.*, 1996). Despite the fact that the mammalian testis, in particular the seminiferous tubules are an immunologically privileged sites such that neither immune cells nor antibodies to sperm components penetrate through Sertoli cell tight-junctions to interfere with intratubular spermatogenic processes (Best and Hill, 1995), we nevertheless anticipated that the antibodies as serum transudate would be available at the rete testis and epididymal sites to interact at the acrosomal region following spermeogenesis. Having successfully identified RCP antibodies on the epididymal spermatozoa in the rat and the voided mature spermatozoa (and seminal plasma) in immunized Bonnet monkeys, it was gratifying to note that such spermatozoa did exhibit greatly impaired motility characteristics *in vitro* in culture. As anticipated, in mating experiments with normal females, immunized male rats showed significant reduction (>85%) in fertility. Examination of the uterine horns by laparotomy on day 8 *post coitus* revealed that a majority of the mated females lacked implantation sites on their uterine horns while a few of the animals harboured degenerative embryonic

inclusions within the pycnotic decidual chambers. Normal ovulation was confirmed by counting the number of corpora leutea on their ovaries. Apparently, antibody binding interfered with either fertilizing ability of the sperm and/or post-fertilization development of early conceptus (Bronson, 1993). Commensurate with this are our findings when the oviducts and uteri of such mated females were flushed on day 5 *post coitus*, i.e. before initiation of implantation. In the majority of such animals, there were no products of fertilization while in the remainder, early embryos when encountered were degenerate due to asynchronous development. Histologically, the testis, epididymis and other accessory sex organs of these immunized male rats did not display gross abnormalities. The plasma testosterone concentrations were also normal, indicating unimpaired functioning of the Leydig cells. Preliminary experiments with male Bonnet monkeys actively immunized with RCM–RCP revealed that anti-RCP antibodies were indeed associated with the acrosomal caps of ejaculated spermatozoa which consequently had impaired motility parameters *in vitro*, particularly in presence of added complement. The semen characteristics appeared otherwise normal with no discernible sperm agglutinating antibodies. In confirmation of observations from the rodent model, active immunization did not alter their circulatory testosterone concentrations. When these male monkeys (n = 4) were repeatedly mated with fertile females during days 10–16 of the ovulatory cycles, none of the latter animals became pregnant. In this ongoing fertility testing exercise it was encouraging to find that a total of 29 fertile cycles in 10 female monkeys have been protected from pregnancy so far. Extended studies are required to confirm these preliminary observations.

Conclusions and future prospects

RCP is an oestrogen-inducible protein, that is evolutionarily conserved and comes into play during procreation of birds, rats and monkeys. It has a physiological role in transporting riboflavin to the developing oocytes. In mammals it transports this essential vitamin to the developing embryo. Recent findings indicate that RCP is also a component of the ovulated oocyte as well as of the trophoblast. It is susceptible to immunointerference without causing any discernible adverse effects on the mother's health. The antifertility effects are reversed when titres of RCP antibodies drop. These findings hold promise of using this unique, reproduction specific protein as an antifertility vaccine in the female primates.

The finding that RCP is a surface-exposed, acrosomal component of mammalian spermatozoa and that a significant reduction in fertilizing ability of spermatozoa occurs following RCP antibody-binding to them indicates that the protein could also be used as a male contraceptive. This concept is particularly attractive since testosterone concentrations and hence libido and accessory gland functions remain unaffected following active immunization with RCP.

It is conceivable that the nature, affinity and concentration of neutralizing antibody population in the reproductive tracts rather than the total antibody repertoire in circulation are crucial for immuno-contraceptive efficacy. In common with other protein antigen-based candidate vaccines for fertility control (Naz *et al.*, 1995), innovative strategies of vaccination to overcome variability in immune response in outbred population, such as appropriate choice of adjuvant cocktail, effective routes and the modes of sustained delivery of the antigen to optimise the duration and magnitude of immune response need to be explored to ensure that a successful RCP-based contraceptive vaccine becomes a reality. Chicken RCP is easily purified to homogeneity from a relatively inexpensive source to be cost-effective for use on a mass scale unlike the problem currently faced with contraceptive vaccines based on reproductive hormones. Additionally, RCP generated by recombinant DNA technology and shown to be immunologically active in curtailing pregnancies in the rat, is currently available on a laboratory scale (Sooryanarayana *et al.*, 1996). Since denatured linearized RCP is effective as the immunogen, shelf-life will be long at ambient temperature.

Studies on immunotopology of chicken RCP have shown that among the antigenic determinants on the protein surface, sequential epitopes can effectively induce immune response that interfere with RCP *in vivo*. Identification of two such B-cell epitopic sequences and the demonstration that they elicit specific neutralizing antibodies capable of curtailing pregnancy in rats and Bonnet monkeys suggest the feasibility of using synthetic peptide-based vaccines for regulating fertility. A systematic search for additional epitopic sequences on RCP surface capable of eliciting neutralizing antibodies might contribute to development of a mixture of synthetic RCP peptides as a vaccine for immunocontraception. Such a search should enable elimination of undesirable but otherwise immunopotent epitopes and suppression sequences. Obviously, additional research efforts in stabilizing their epitopic conformation by peptide engineering to enhance the magnitude and the duration of the immune response are desirable prior to realization of

these goals. Replacement of traditional protein carriers such as DT for conjugation by synthetic T- non-B peptides of 'promiscuous' nature may help to enhance immunogenicity in low responders in an outbred population with different genetic background. In common with other candidate vaccines for immunocontraception, it is obligatory to ensure the long-term safety of RCP as an antifertility vaccine by detailed toxicological and immunopathological investigations. It is hoped that based on basic studies, this novel reproductive protein (or specific epitopic sequences thereof) with contraceptive potential in both the male and female, would contribute to the development of a cocktail approach to immunocontraception. Such a vaccine-based method of fertility control could become a cost-effective, socially acceptable alternative to traditional methods of fertility regulation.

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