Epididymis as a target for contraception

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Advantage of using a vaccine based on sperm antigens is that it can be used both in males and females as individuals who have antisperm antibodies are usually infertile but otherwise healthy. Several sperm specific antigens identified as prospective candidates for immun contraception are of testicular origin. For the purpose of immun contraception it may be desirable not to disrupt spermatogenesis and testicular function. Concept of post testicular maturation of spermatozoa has been very well established. During post testicular voyage spermatozoa undergo a series of complex and sequential events which transforms the immature immotile spermatozoa into mature sperm. Acquisition of functional maturity is necessary for progressive motility, zona pellucida recognition culminating in sperm egg binding. Importance of epididymal maturation is highlighted by the fact that high percentage of male infertility in human originates from the malfunction of the epididymis.

The epididymis has also shown to be involved in sperm storage and provides an adequate environment for final maturation of the sperm. It provides a conductive microenvironment by virtue of which the spermatozoa are protected during the storage. In view of this it is imperative that more attention needs to be focused on epididymal antigens. The information obtained will enable us to identify epididymal antigens relevant to fertility and also help in infertility diagnosis.

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There is an urgent need to provide safe and sustained effective fertility control for the world population1. More and improved contraceptive options is the need of the hour. This is especially relevant to males who currently have only four limited options: abstinence, withdrawal, condoms and vasectomy. Of these vasectomy is essentially irreversible and is not the preferred method for men who wish to father a child at a later date. Although the results from studies involving hormonal methods for male contraception are convincing2 it has not yet yielded a marketable product. Vaccine approach for contraception would definitely be a valuable addition to the existing armamentarium of different approaches used for family planning. Vaccine based on sperm antigen is very promising and, therefore, in spite of no real breakthrough, they are being actively pursued. The targets have been sperm antigens coming either from the testis or the epididymis. Realizing the feasibility of this approach and the need for additional methods for contraception National Institute for Child Health and Human Development (NICHHD) convened a workshop to identify novel strategies involving testicular and epididymal antigens for developing a male contraceptive in the 21st century2.

Testis as a target for contraception

Testis is the primary organ which produces spermatozoa and, therefore, has been considered to be a good target for contraception. It is indicated that this could be done by direct interference at various stages of spermatogenesis such as 1) interference with meiosis and particularly the cell cycle check points 2) disruption of RNA protein interaction (post transcriptional control) 3) disruption of junctional complexes between sertoli cells and germ cells and 4) attacking different testicular proteins5. Number of testis/sperm specific proteins have been identified using different approaches and several of them have also been cloned and sequenced. The current status, application, relative merits and immunogenicity of these antigens has been extensively reviewed6.

Epididymis as a target for contraception

Post testicular contraception could be achieved by interfering pharmacologically or immunologically with the process of sperm maturation in the epididymis. Maturational changes which the sperm undergoes during epididymal transit have been shown to be prerequisites for successful fertilization. It is suggested that theoretically these may be interrupted at different sites. It is possible that modifying the pattern of epididymally secreted proteins may change the optimal environment for maturation and storage of sperm7.
Advantages of epididymal antigens for contraception

Vaccination of males for contraception has not been possible beyond preclinical levels for a variety of concerns. Testis is an immunologically privileged site because of the blood-testis barrier. It may not be advisable to immunize men with testicular sperm antigens as it may lead to irreversible testicular damage. It is suggested that antibodies raised against antigens present exclusively post-testicularly may be less likely to induce autoimmune orchitis. There are definite advantages in targeting the epididymis for contraception. Firstly the onset of infertility and also its reversal has been shown to be far quicker than any agent attacking the testicular production of spermatozoa and secondly, as maturing cells are targeted, damage to the genetic material, a possible sequel of effect on dividing germ cells is avoided. It would also avoid endocrine impairment of libido.

In order to pursue the epididymis as a target for contraception there is a need to understand the different roles played by epididymal proteins in sperm maturation, and sperm protection during storage. The new technologies such as cDNA arrays and proteomics, will help us identify the various proteins involved in sperm maturation immunological protection as well as those possessing antimicrobial activity which help in sperm protection. Once such molecules are identified we will be able to understand the importance and composition of the epididymal milieu which plays a major role in acquisition of fertility, motility.

Role of epididymis in sperm maturation

Epididymis was earlier thought to be just a passive channel through which spermatozoa travel to be stored before being ejaculated. The concept of post-testicular maturation in mammalian spermatozoa evolved as a result of the pioneering studies of Benoit and Young. But it was Bedford and Orgebin Crist who initiated depth investigations and demonstrated that, sperm which were prevented from passing along the epididymal duct were viable but did not acquire full fertilizing capacity. Large amount of information has become available after 1960, about the structure, biochemical properties and functions of this organ. It has been shown that in the epididymis there is complexity in the cellular properties, heterogeneity, region specific expression and spatial and temporal organization of proteins. All these complexities make this organ very dynamic. It is now clear that the maturation of spermatozoa and the acquisition of motility and fertilizing ability do not result from a passive journey of spermatozoa but rather as a result of exposure to and active interaction with the luminal contents of different epididymal regions.

Membrane remodelling

During its epididymal sojourn the sperm plasma membrane undergoes intense changes both in protein composition and localization on the gamete. The sperm plasma membrane is originally derived from spermatogonia/spermatocytes in the testis but undergoes extensive remodeling during spermiogenesis in the testis, maturation in the epididymis and capacitation in the female genital tract. The epididymal maturation events take place due to the microenvironment formed by the luminal contents of the epididymal duct. Remodeling of the membrane could be brought about either by uptake of secreted epididymal proteins, or processing of existing or acquired proteins. Processing of existing or acquired proteins occurs as a result of some glycan modifying enzymes such as glycosidas and glycosyltransferases or protein modifying enzymes such as endo-proteases and protease inhibitors that are found freely in the luminal fluid or are associated with sperm plasma membrane. A deficiency of these enzymes has been shown to be responsible for male infertility. These remodeling mechanisms lead to acquisition of specific functions by different domains of the spermatozona during epididymal transit.

Repositioning of protein components to membrane domains occurs as seen in family of proteins such as ADAM (proteins with a disintegrin and metalloproteinase like domain) for example fertilin (PH20) or membrane proteins such as MDC (protein with a Metalloproteinase, a disintegrin-like and a cysteine-rich domain) for example 2B1. Relocation of proteins can be seen across different domains in proteins which belong to immunoglobulin super family for example, C69 where the shift is from principal piece to midpiece.

Every domain of the sperm is endowed with a specific function. Head of sperm comprises of acrosome and post acrosomal region. Acrosome is involved in primary binding to egg plasma membrane. Equatorial/post acrosomal region is involved in secondary binding leading to fusion. The flagellum is divided into midpiece, principal piece and end piece. Midpiece is associated with generation of
energy (ATP), principal piece which contains the axoneme and fibrous sheath is responsible for flagellar flexibility and motility. Pro tease of the fibrous sheath are implicated in signaling pathway in spermatozones and in glycology pathway. Protein DE which has been localized in the acrosome region is shown to have a role in sperm egg fusion. A 26kDa protein localized in the flagella has been shown to have a role in motility.

**Protective role of the epididymis**

Epididymis plays a very important role in protection of spermatozoa but is the least studied aspect. Spermatozoa spend many days traversing the long epididymal duct and are faced with a constantly changing microenvironment. Epididymis provides conductive microenvironment by rapidly eliminating the harmful metabolic by products. It also protects spermatozoa by blood epididymis barrier provided by tight junctions between the principal cells. This barrier not only protects the spermatozoa from external non conductive environment but also prevents the access to the immune system. This barrier is selective and does not allow passage of high molecular weight compounds like L-glucose, insulin, BSA but readily allows the passage of water, D-glucose and amino acids showing that the blood epididymis barrier helps in the maintenance of conductive microenvironment for the spermatozoa.

Epididymis has also been shown to play a role in protection of spermatozoa from free radicals and recognition and elimination of defective spermatozoa. Epididymal spermatozoa are extremely vulnerable to oxidative stress. To overcome this problem epididymis has a rich source of an anti oxidant enzyme that scavenges any excess reactive oxygen metabolite released by the spermatozoa during epididymal transit. The caput epididymis also secretes glutathione peroxidase which gets intimately associated with the sperm surface where it serves to remove any H2O2 generated by these cells as a consequence of SOD action. Molecules such as glutathione peroxidase, catalase, superoxide dismutase have been shown to protect sperm in the epididymis from damage due to reactive oxygen species. Studies on expression of mRNA for B - Defensin-1 and 2 in the initial segment and caput epididymis suggest a role for the epididymis in antimicrobial protection. Quality control of male gametes is ensured by an active apoptotic pathway present in spermatogenic lineage and in mature sperm of mice and men.

Though ubiquitin was previously detected in human epididymal cells and seminal plasma, its importance in quality control of fertility was not indicated. Although both normal and defective sperm carry constitutively ubiquitinated substrates it is only the defective ones that get surface ubiquitinated during epididymal passage. Sutovsky and coworkers have shown surface ubiquitination of sperm and subsequent phagocytosis by epididymal epithelial cells.

**Role of epididymal proteins in reproduction**

There are several direct and indirect evidences which underline the importance of epididymal proteins in reproduction. Number of clinical evidences show the correlation between abnormalities or disturbances in the epididymal secrections and infertility. In fact high percentage of male infertility in human is believed to originate from the malfunction of the epididymis. Incomplete sperm maturation within the epididymis has been suggested to be the cause of total failure of sperm binding to zona in unsuccessful human in vitro fertilization cases. Vasectomy also has shown to cause irreversible damage to epididymis and is thought to be one of the causes of infertility even after vasovasostomy. Human P34H1 protein which is involved in sperm-zona pellucida interaction is found to be reduced in certain cases of idiopathic infertility. P25b and P21b proteins (bull homologues of human P34H1) are associated with sub-fertility and their expression is markedly low in subfertile bull. It is speculated that epididymal antigens play a predominant role in several cases of human infertility. All these evidences point to the important role of the epididymis in bestowing on the sperm motility and fertilizing ability. In view of these data it is clear that epididymal proteins have a vital role in maturation of spermatozoa, leading to successful reproduction and, therefore, more attention needs to be focused on identification of epididymal antigens.

Several epididymal proteins have been identified and studied for their contribution towards sperm maturation. However, many more still remain to be identified and their functions need to be ascertained. Identification of newer epididymal proteins and annotating their function will help in understanding the mechanism of sperm maturation and the sequence of events therein. This will further help in selecting epididymal targets for contraception which will specifically alter the ability of the sperm to fertilize.
without any side effects. Hence understanding the epididymis in more details will help in treating certain types of male infertility and also to develop male contraceptive agents.

Different approaches have been exploited for identification of epididymal proteins such as use of lectins, subtractive screening of the epididymal cDNA library, use of the expressed sequence tag proteomics and neonatal tolerization. Neonatal tolerization is a powerful tool for raising monoclonal antibodies to rare or weakly immunogenic antigens. This approach has been used by several investigators for generating monoclonal antibodies to rare or less immunogenic antigens. In this approach once a state of tolerance to an antigen is established, the tolerized animals could be subsequently immunized with a crude preparation of the potential antigen (immunogen). By inducing the immune tolerance to the tolerogen, the immune system will generate an immune response to only those epitopes not included in the tolerogen preparation. This approach increases the probability of obtaining antibodies to functionally significant components that may be weak immunogens.

Identification of epididymal proteins by conventional immunization followed by hybridoma technology has not been very successful, probably because the testicular proteins are more immunogenic than the epididymal proteins. Therefore, the alternate method of, “Neonatal tolerization” also called as subtractive immunization first reported in 1991 was exploited by us with slight modifications. Using this method we, were able to generate an immune response to epididymal antigens in BALB/c mice. In our protocol (Fig 1a) animals were tolerized to testicular protein (tolerogen) at birth and were then immunized with epididymal sperm protein (immunogen) for raising specific immune response. These studies demonstrated that neonatal tolerization with testicular proteins followed by immunization with epididymal sperm proteins enhanced the production of antibodies to epididymal proteins. Serum from these animals localized proteins only in corpus epithelium and sperm from corpus and cauda epididymis but showed no reactivity with testis (Fig 1b). Using sera from these neonatally tolerized and immunized mice, we identified a dominant epididymis specific protein of molecular weight approximately 27 kDa which was used to raise polyclonal antibodies in rabbit. The polyclonal antibody was very specific to epididymis as seen by ELISA, Western blot and IHC. The antibody identified a 27 kDa protein from homogenates of epididymis as well as sperm from corpus and cauda region. The protein identified was present on the midpiece as seen by indirect immunofluorescence and was androgen regulated and was also developmentally regulated. These results indicated that this protein is secreted mainly in the corpus and acquired by the spermatozoa during passage through these regions. The presence of the protein in corpus and cauda may be due to its secretion by the principal cells of the corpus. The principal cells of the corpus have a well developed endoplasmic reticulum and Golgi apparatus, which indicates that they are involved in active protein synthesis. There are some reports where principal cells of epididymis have been shown to incorporate labeled amino acids and transport radiolabelled molecules through the cell. Principal cells have been shown to be actively involved in the physiological functions of the epididymis, involving endocytosis and secretion. It is reported that proteins secreted by principal cells may interact with spermatozoa in the lumen of epididymal duct and enable spermatozoa to develop motility and fertility. It has been shown that corpus/cauda junction plays a major role in sperm maturation. Fertilization rate is shown to be higher in spermatozoa from corpus than from caput as seen in vitro fertilization. Sperm motility and pregnancy rates were also found to be significantly improved when the vas was surgically joined to the corpus in those patients who received specific tubule vasectomy or were infertile male and female or vasectomized male have also proved to be a good source of antisperm antibody for characterization of sperm antigen involved in fertility. Using sera from infertile male and female or vasectomized male we identified a 28 kDa sperm protein of epididymal origin and suggested that autoimmun e infertility might represent a response to the epididymal rather than testicular sperm. He further suggested that monoclonal antibodies raised to such unique and immunologically accessible sperm coating antigens in
the epididymis rather than in the testis would seem to present a theoretical solution to male infertility.

We generated large number of hybridomas, using Balbc mice which were tolerized to testicular proteins and immunized with epididymal sperm proteins. Majority of clones showed high reactivity with epididymal sperm protein while a small number were found to react with testicular sperm protein. This indicated that neonates were successfully tolerized to testicular antigen and mounted immune response to epididymal proteins. Immunofluorescent localization using polyclonal serum from the TI mouse used for fusion localized antigens in different regions of the sperm such as acrosome, post acrosome, equator, midpiece and tail. This indicated that epididymal proteins are located on different regions of the sperm and are likely to play domain specific roles such as sperm-egg interaction, acrosome reaction and motility which are essential for fertilization. It was interesting to note that the pattern of IIF localization was seen to be identical in both gluteraldehyde fixed spermatozoa smeared on glass slide as well as spermatozoa in

**Neonatal Tolerization-Immunization Protocol**

- Mice injected with tolerogen (testicular protein)  Day 0
- Mice injected with tolerogen (testicular protein)  Day 5
- Mice bled retro orbitally to check reactivity with testicular proteins  Day 21
- Immunized with epididymal sperm protein
- Two boosters at 2 weeks interval
- Bled retro orbitally to check titer against epididymal and testicular sperm proteins

Fig. 1(a) — Flow chart showing protocol for neonatal tolerization and immunization

![Fig. 1(b) — Immunohistochemical localization of antigens using serum from neonatally tolerized immunized mouse. Testicular section shows no staining. The corpus section shows localization in the supranuclear region of the epithelium and on spermatozoa. The cauda epididymal section shows no staining in the epithelium but strong staining on the spermatozoa in the lumen]
**Fig. 2** — Immunohistochemical and functional characterization of antigen using monoclonal antibodies.

a1-a4: Representative picture of immunohistochemical localization in different regions of epididymis using a monoclonal antibody. (a1) Proximal caput shows no staining. (a2) Distal caput shows some localization. (a3) Corpus epithelium shows strong localization. Spermatozoa in the lumen are also stained. (a4) Cauda epididymal region shows localization on ciliary lining and spermatozoa.

b1-b3: Immunofluorescent localization of antigens on spermatozoa using different monoclonal antibodies. (b1) MAb V2C4E2 stains acrosomal region. (b2) MAb V3C8, V3F4F4 stain post acrosomal and equatorial region. (b3) MAb V1B8E10, V3C10 stain mid-piece region.

c1-c2: Agglutination pattern using different mAbs. (c1) Radial pattern of agglutination seen with mAb V2C4E2, V3C8, V3F4F4. (c2) Comet shape pattern of agglutination seen with mAb V1B8E10, V3C10.

This observation along with the agglutination pattern indicated that the proteins identified by the monoclonal antibodies are on the surface of sperm. Surface localization of sperm antigens is one of the criteria for ideal contraceptive targets.

Earlier studies from our laboratory have shown that passive immunization with antibodies to 26 kDa epididymal specific protein were very effective in bringing about antifertility effect in both female and male mice. In case of the female mice, the antibody showed a dose dependent reduction in the antifertility effect and was found to be effective only before fertilization. Whereas, in case of male mice, the antibody was able to enter the epididymis within 24 hr and inhibit sperm maturation and sperm function. Fertility was reduced to 100% after about 5 days of antibody administration. Histologically, the effect was partially reversed after a week and completely reversed after two weeks. These studies clearly indicated that where spermatogenesis is unaffected, the recovery of fertility was faster.

Advantage of using a vaccine based on sperm antigen is that it can be successfully used both in males and females, as individuals who have antisperm antibodies are usually infertile but otherwise healthy. Of these antigens the epididymal antigens make better candidates for immunocontraception as they inhibit only post testicular maturation of spermatozoa, making them infertile without affecting
testicular function. It is suggested that antigens that do not cause testicular pathology but can access the epididymal lumen and eliminate the spermatozoa or impede their function, with potential reversible antifertility effect would be worthy of consideration for immunocontraception.

Post testicular approaches to male contraception are yet in its infancy and have not reached the clinical phase. It would be necessary to investigate the molecular physiology of sperm maturation and epididymal function. This would be possible only if we identify target molecules. Once this is achieved these molecules could then be blocked by specific pharmacological agents with a rapid onset of action.

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