

Review

Identification of sperm immunoreactive antigens for immunocontraceptive purposes: a review

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Abstract

Antisperm antibodies (ASA) may be a reason of infertility in some individuals. They may affect pre- as well as post-fertilization stages of the reproductive process. There is ongoing progress in the identification of sperm antigens related to fertilization. The employed methods for this purpose include recombinant DNA technology and the most advanced proteomic analysis. This paper enlists the different approaches undertaken in order to identify and characterize the immunoreactive sperm antigens. We have mainly focused on those, which have been already studied in regard of their immunocontraceptive potential, although it has been impossible to include all published data concerning the topic in a single article. Few novel sperm auto- and isoantigens, discovered recently, have also been reviewed even if their role in fertilization has not been yet established.

Introduction

Infertility occurs in one out of the five couples of the reproductive age. Some of these individuals (males and females) possess antisperm antibodies in blood and reproductive tract secretions. ASA may influence pre-fertilization stages of the reproduction process (sperm agglutination and/or immobilization, sperm-oocyte interactions) and they can inhibit the development of the post-fertilization zygotes.

Identification and characterization of antigens present on sperm cells is crucial for understanding their cognate ASA relationship with infertility. However, due to the complexity of polyclonal sera, containing ASA interacting in most of the cases with multiple sperm components (either specifically or by molecular mimicry), the identification of a single immunodominant sperm antigen seems to be impossible.

Interaction between antibodies and some of the sperm membrane antigenic moieties may be regarded as a main reason of immune infertility, because in live sperm cells ASA are not able to penetrate through the plasmalemma (except from the acrosomal antigens that appear on the sperm surface after the acrosomal reaction). On the other hand, detailed knowledge of the nature of sperm antigens, engaged in immune reactions may be helpful in developing technology for contraceptive vaccination, based on sperm – specific components, for regulation of fertility in humans as well as in domestic and wild animals populations [1,2].

The administration of sperm antigenic cocktails, prepared from whole semen specimens for immunocontraceptive purposes is unacceptable due to the reported hypersensitivity reactions against seminal plasma components [3] and molecular mimicry with various somatic cells [4].

ASA, despite the presence within the organism for years do not exert any harmful effect on patients, except for their infertility. One must remember, however, that not all antisperm antibodies will alter sperm function, either because the cognate antigen is not involved in the process of fertilization or because the antibodies do not bind to the functional domain of the antigen. It is well known from monoclonal antibodies that the antibody-binding region of the antigen may be different from the region being active in metabolic processes [5].

In summary, the selection of a certain sperm antigen(s) for the development of a contraceptive vaccine is limited by its specificity, participation in the fertilization process and its potential to induce a high sperm-specific antibody titer in the genital tract.

Antibody identification of sperm antigens

Many methods can be used to identify of cognate antigens of ASA. Therefore, antibodies secreted into body fluids of spontaneously *in vivo* sperm-sensitized males and females, were employed for Western immunoblotting and immunoprecipitation studies with sperm antigenic extracts [6-11]. The produced data, indicating the molecular weight of relevant sperm antigens were, however, conflicting, probably due to differences either concerning a methodology or selection of the studied populations of the ASA-positive individuals. Besides, the molecular weights of immunodominant entities were insufficient for their subsequent characterization, including their primary structure, tissue specificity and determination of the immunogenic regions for subsequent cloning.

In addition, the use of circulating ASA may not be the most appropriate way to identify the antigens really involved in fertility, as the major antigens defined by circulating ASA differ from the antigens recognized by sperm-bound antibodies [12]. Thus, antibodies reacting with the surface of human spermatozoa were isolated and an enhanced chemiluminescence immunoblotting technique was used for analysis of sperm antigens recognized by antibodies eluted from the surface of spermatozoa obtained from infertile men with unsuccessful *in vitro* fertilization [13]. In this study, 2 protein zones: 37/36 kDa and 19/18 kDa were electroeluted from the preparative slab gels and were used for biochemical characterization and the production of polyclonal antibodies in rabbits. Previously, [12] these proteins were recognized by antibodies of the great majority of infertile individuals. Isoelectric focusing showed that these peptides (designated as P36 and P18) consisted of several polypeptides of the pI 4.5-8.3 for P36 and 4.75 to 5.9 and 7.0 - 10.3 for P18. Human ASA reacted with few spots with pI 5.0 - 6.0 for P36 and several spots with pI 8.3 - 10.3 for P18. Immunofluorescent studies revealed the presence of these pep-

tides on sperm heads. Anti P36 antibodies reduced the binding and penetration of zona-free hamster oocytes by human sperm. Anti P18 antibodies reduced sperm-oocyte penetration but did not significantly affect sperm binding.

In most of the published Western immunoblotting studies the reactions of sperm antigens with sera of fertile patients were not analyzed. Hence, it was impossible to reveal the role of these antigens in the fertilization process.

Monoclonal antibodies may be also applied for identification, isolation and characterization of sperm antigens. Although many antigens have been identified by means of monoclonal antibodies [14-19], the identification of human homologues of antigens originally identified in an animal model is often difficult as well as the purification of sufficient natural product from human tissues for detailed biochemical characterization and studying the fertilization process in primates [20]. A solution could be a combination of monoclonal antibodies (or ASA derived from sera of infertile individuals as well as sperm-sensitized animals) with testis cDNA libraries for direct identification of sperm-specific antigens. The advantage of this approach is the direct confirmation of tissue-specificity of relevant cDNA fragments. Since recombinant proteins are lacking the carbohydrate moieties, their further analysis is much simpler. Even if this approach is limited by the fact that during maturation the sperm plasma membrane undergoes changes (resulting in the appearance of new antigenic components acquired in the epididymis [21]), it has been successfully applied for identification of some novel sperm antigens.

cDNA library screening

Sera of ASA-positive infertile patients allowed identification of a novel 56 kDa antigen X-1 (AgX-1) from the human λ gt11 cDNA library [22].

In another study, the FA-1 [23] MoAb was used for a screening of murine testis lambda gt11 cDNA expression library [24]. The phage DNA of a positive clone was subcloned and subsequently sequenced. Search in databases did not identify any known nucleotide/amino acid (nt/aa) sequences having homology with FA-1 cDNA or its deduced aa sequence, suggesting a novel protein. The recombinant FA-1 (rFA-1) was obtained and investigated for its immunocontraceptive potential in mice [25]. The immunization of female mice with the murine rFA-1 antigen resulted in significant reduction of fertility that was reversible when the antibody titer declined to the normal level. Murine anti-rFA-1 antibodies blocked murine binding to ZP and IVF in murine oocytes. Anti-rFA-1 antibodies specifically reacted with a protein band (of approximately 47 kDa) only in the testis extract, in the

Western blotting procedure. These findings make the FA-1 antigen one of promising candidates for immun contraception studies. A human homologue of the murine FA-1 cDNA has been also obtained and characterized, proving its novelty and expression limited exclusively to human testis on the mRNA as well as on the protein level [26]. The antibody to the recombinant human FA-1 antigen caused a significant ($P < 0.01$) and concentration – dependent inhibition of human sperm capacitation and acrosome reaction by blocking tyrosine phosphorylation of the FA-1 antigen. The FA-1 antigen has been already applied in a clinical trial for adsorption of IgA and IgG autoantibodies from the surface of sperm cells of immunofertile men thus improving their fertilizing potential [27].

One of the antigens identified by a serum sample from an infertile ASA-positive women, is the BS-17 antigen [28]. Immunofluorescent studies with human, rat, rabbit, mouse and hamster spermatozoa showed localization of this antigen on the surface of the acrosomal region of mammalian spermatozoa with variable (species-dependent) localization in the sperm tail. The human testis λ gt11 cDNA expression library was probed with polyclonal anti-BS-17 antibodies. The positive clone yielded a cDNA fragment, consisting of 758 base pairs with an open reading frame of 558 bp, encoding a polypeptide composed of 186 aa residues, with high homology to calpastatin [29]. *In situ* hybridization, with human testis, showed that calpastatin mRNA transcription had occurred only in spermatids [30]. The inhibition of calpastatin leads to a premature acrosome reaction [31]. Polyclonal antibodies to the BS-17 antigen inhibited human sperm from penetrating and fertilizing zona-free hamster oocytes [28]. The suggested mechanism of this inhibition involved destabilization of the calpastatin-calpain complex by the anti-B-17 antibodies, thus calpain (calcium – dependent cysteine endopeptidase) could trigger the sperm acrosome reaction before it would reach the ovum, resulting in deterioration of the sperm fertilizing capability.

In another study, the human testis λ gt11 cDNA expression library was screened with polyclonal antibodies to the EP-20 glycoprotein [32]. A positive clone yielded a cDNA (designated as HED-2), encoding a polypeptide consisting of 493 aa and having 99% of aa sequence identity with zyxin, a constituent of the adhesive matrix of cell-cell and cell-basement membrane junctions, involved in the intercellular exchange and transport of nutrients and metabolites, and binding of ligands to the plasma membrane [33,34]. HED-2 protein, present in Sertoli cells, might participate in the modulation of the differentiation of spermatogonia to spermatozoa.

Naz and Zhu [35] screened the mouse λ gt11 library with polyclonal sera obtained after immunization of mice with human sperm antigens contained in bands belonging to the Mw of 14–18 kDa, excised from the polyacrylamide gel after SDS-PAGE. These antibodies recognized a cDNA clone coding for an antigen designated as NZ-1. Neither cDNA nor the amino acid (aa) sequence of its protein product matched any known cDNA/aa sequence. Northern hybridization with mRNA isolated from 11 mice tissues (liver, brain, lungs, kidney etc.) revealed the expression of this sequence only in the testis. Then, it was postulated that the NZ-1 antigen was a novel, sperm-specific antigen. Immunization of mice with the recombinant NZ-1 antigen resulted in reducing their fertility rates.

NZ-2 antigen was identified during screening of human testis cDNA library with antibodies contained in sera of rabbits immunized with human sperm antigens contained in the acrylamide gel bands (corresponding to 14–18 kDa), excised from the slab gels after SDS-PAGE. In immunoblotting with human sperm extract these sera recognized an antigen of approximately 20 kDa. Computer assisted analysis of the positive cDNA clone sequence revealed that this novel antigen (NZ-2) was coded in the human chromosome 7 [53].

The cloning and characterization of a novel, testis-specific, X-linked gene product, expressed exclusively in haploid spermatids has been also reported [37]. In this study cDNA clones were isolated from a human testis λ ZAP cDNA library and probed with mouse monoclonal antisperm antibodies. This yielded a clone designated SPAN-Xa that allowed preparation of a DIG-labeled SPAN-Xa cDNA probe. The latter was used to screen a human testis λ DR2 cDNA library and resulted in obtaining a second clone, designated SPAN-Xb. The SPAN-Xa and SPAN-Xb cDNA sequences were 92.2% identical and their protein derivatives had 83.5% homology. SPAN-X antigen is localized to nuclear craters and/or cytoplasmic droplets of formaldehyde-fixed, methanol-permeabilized human spermatozoa [37].

The FliTrx phage display library was screened with solubilized human zona pellucida (ZP) in order to identify peptide sequences that might be involved in ZP binding [37]. Twelve clones with strong binding responded. The clones contained three aa sequences, designated as SNR12, GHR12 and YLP12, reacted with 50% frequency. Computer software allowed designing a consensus sequence (Consensus17), derived from all the clones. All four sequences did not have any known nt/aa homologues in the sequence databases. Peptides corresponding to YLP12 and Consensus17 sequences inhibited sperm binding with the human ZP in the hemizona assay. The inhibition was dose-dependent (stronger inhibition in higher con-

centrations) and occurred only when the hemizonae were incubated with the peptides prior to sperm. The YLP12 was a stronger inhibitor, so further studies focused on this particular sequence. Fabs against it were produced and applied for immunoprecipitation with solubilized 10 different human tissues. As a result, YLP12 Fabs specifically recognized a protein band of about 72 kDa only in the testis. The antigen was immunolocalized in the acrosomal region of the methanol-fixed human sperm [37]. It was also indicated that YLP12 Fabs significantly reduced the acrosome reaction [38]. Sera and SP samples of the immune infertile men were ELISA tested for the reactivity with the synthetic YLP12. Out of 15 immunoinfertile sera samples, 46% were positive for IgM, 73% were positive for IgG and 40% for IgA. Among 30 immunoinfertile SP samples 10% were positive for IgM, 20% were positive for IgG and 43% for IgA. Serum and SP samples of the fertile individuals did not reveal positive reactions with the YLP12 peptide [39]. In this study, antipeptide antibodies eluted from sera and SP samples of infertile individuals reacted with a specific band of about 72 kDa in the human testis, in immunoblotting.

YLP12 was further studied for its immunocontraceptive potential in the mouse model [40]. A vaccine was obtained by conjugating the synthetic YLP12 peptide with the binding domain of recombinant cholera toxin and its effect on fertility rates and tissue specificity was examined. In this study, intramuscularly and intranasal immunization of the female mice caused 70,3 and 61,4% overall reduction in fertility, respectively. Fertility of the sensitized animals could be regained either voluntarily (by intravaginal administration of the YLP12 peptide) or involuntary (by natural declining of the antibody concentration in sera). Immunoblotting analysis revealed that antibodies resulting from vaccination were tissue-specific and recognized a specific protein band of about 72 kDa in the testis extract and a band of approximately 50 kDa in the sperm extract, the entities were not observed in the extracts of 10 murine somatic tissues. The authors suggested that the YLP12 peptide sequence was a part of a 72 kDa protein, that is synthesized during spermatogenesis in the testis and then is modified or cleaved during epididymal transit. The 50 kDa antigen is localized on the surface of the acrosome and tail of mature sperm cells. In conclusion, the properties of the YLP12 peptide (i.e. its capability to induce sperm-specific response, long-lasting and reversible contraceptive effect, with a complete regain of fertility) make it an attractive candidate for immunocontraception.

Human testis cDNA – ZAP II library screening allowed also to discover another novel and testis-specific antigen, named a contraceptive vaccinogen (CV) [41]. The recombinant form of this antigen was obtained and specific anti-

bodies produced. *In vitro* studies showed inhibition of human sperm from penetration of zona-free hamster oocytes and human sperm binding to human zona pellucida. The CV antigen is also expressed in murine sperm [42] suggesting a feasibility of animal model for studying its immunocontraceptive potential.

Two-dimensional electrophoresis

In yet another approach, high resolution two-dimensional electrophoresis with separation of sperm antigens in the first dimension by either IEF or NEPHGE followed by SDS-PAGE and Western blotting can be applied to study sperm – immune system interactions. Two-dimensional electrophoresis, according to some authors is currently the most powerful and informative tool available for analysis of complex mixtures of polypeptides [43].

In the study of Naaby-Hansen et al. [44] a repertoire of 1397 silver-stained proteins has been obtained, digitized, catalogued and characterized by the Mw, pI and the shape of spots. Some of them were identified as sperm coating antigens, derived from SP.

These data were afterwards employed for the identification of human sperm antigens reacting with the sera of infertile women having sperm immobilizing antibodies [43]. Percoll-purified human sperm proteins were separated by IEF followed by SDS-PAGE and transferred to nitrocellulose. Then, the membranes were immunoblotted with either SI-positive or SI-negative serum samples. Then, Mw and pI identified the subset of prominent immunoreactive sperm surface antigens: (32.6, 5.9), (35.6, 6.6), (58.6, 4.9), (93.8, 5.5).

The study performed by Shetty et al. [45] also employed 2-D gel electrophoresis, with the separation of human sperm proteins in the first dimension by IEF or NEPHGE, followed by PAGE. Sera of infertile male and female patients were chosen for further study on the basis of their high IBT reactivity (over 60%). In this study 6 auto- and isoantigens were appointed as possibly being relevant to infertility (kDa, pI): (34.0, 4.2), (38.0, 4.3), (60.0, 6.2), (60.0, 6.4), (88.2, 4.0), (140.0, 4.2). These antigens differed from the ones identified by the SI-positive sera used by Shibahara et al. [43], probably because ASA detected by means of IBT may not be associated with infertility and could differ from SI-ASA. Shibahara and colleagues [43] suggested that sperm surface antigens, corresponding to SI antigens may be considered as the ideal targets in developing immunocontraceptives. However, the detected spots representing candidate sperm surface antigens must be sequenced, their cognate cDNA cloned and tissue specificity established before immunocontraceptive trials.

A novel human sperm membrane antigen, SAMP32 (sperm acrosomal membrane-associated protein 32) was discovered from two-dimensional analysis of human sperm extracts containing hydrophobic proteins that were partitioned into Triton X-114 [46]. Among several dozen hydrophobic sperm proteins microsequenced by mass spectrometry, 4 spots shared the same peptide sequences. Cloning the corresponding cDNA it was revealed that they were products of a single gene, localized to the chromosome 6 in human. Further investigations revealed that SAMP32 expression was testis-specific, with the expression to Golgi phase round spermatids and subsequent stages of acrosome biogenesis, and in mature spermatozoa it was localized on the inner acrosomal membrane in the principal and the equatorial segments of the sperm acrosome. Before acrosome formation, SAMP32 expression did not occur. SAMP32 is an isoantigen in humans. Rat antibodies to the recombinant SAMP32 protein significantly suppressed the binding and the fusion of capacitated human spermatozoa with zona-free hamster eggs in comparison to preimmune serum. Serum from an ASA-positive infertile man strongly reacted with the recombinant SAMP32 antigen, suggesting that it might be one of the antigens related to immune infertility and (on the basis of *in vitro* studies) that it might have a role either in binding and fusion of sperm with the oolemma or in sperm internalization [47].

Recently, a novel epididymis – specific glycoprotein, E-3 was identified in Lewis rats by means of 2-D gel electrophoresis, followed by immunoblotting with rat hyperimmune sera raised against isologous rat sperm [47]. The E-3 antigen (28 kDa, pI 3.5) is present in the epididymis but not in testis and other tissues (as revealed by Northern blotting). Indirect immunofluorescence showed its presence on the sperm tail with less intense staining on the sperm head. The E-3 protein sequence did not match any known proteins from the databank but resembled other newly discovered proteins, e.g. ESP13.2 from human and macaque as well as 2 putative novel human proteins, detected by sequencing of the human genome [47]. Its defensin-like motif suggests its role in protecting sperm and the epididymis from microbial infections.

Bohring et al. [48] performed 2-D electrophoresis of highly enriched sperm membrane proteins and immunoblotted them with 20 MAR-positive SP samples of infertile individuals. Overall, 18 immunoreactive antigens were obtained, out of which 6 were analyzed using matrix-assisted laser desorption ionization- mass spectrometry (MALDI-MS) and peptide matching. Among the studied SP samples, 95% reacted with the disulphide isomerase ER60, 90% recognized the inactive form of caspase-3 and 75% of the SP samples reacted with the spots identified as heat shock proteins (HSP70 and HSP70.2). The other

entities recognized by ASA of infertile men reacted with two subunits of the proteasome (C2 and zeta chain). According to the authors, however, additional experiments with using specific antibodies would be necessary to demonstrate that the protein spots characterized are indeed related to the proteins identified by MALDI-MS. Their role in sperm function must be also determined.

Two-dimensional electrophoresis analysis was also useful for identification of targets at the intersection between the calcium and protein tyrosine kinase signal transduction pathways during capacitation of human spermatozoa [49]. This strategy resulted in the identification of another, unique to spermatogenic lineage, 86 kDa antigen, a calcium-binding tyrosine phosphorylation-regulated protein (CABYR) [49]. Its gene is localized to the human chromosome 18 and consists of 5 exons. In methanol – fixed human sperm it was localized by indirect immunofluorescence on the principal piece of the sperm tail. Electron microscopy analysis revealed its distribution over the fibrous sheath compartment, including the surface of the longitudinal columns and ribs. Calcium binding capacity of CABYR may represent one molecular mechanism for capacitation and hyperactivation [49].

Conclusions

Many studies have been performed so far on candidate antigens from male and female gametes in order to demonstrate their ability to induce auto and/or isoimmune response interfering with fertility [50]. Clearly, several antigens of gametogenic origin have been successfully applied to induce infertility in experimental animals. Because no single predominant target of polyclonal antisperm antibodies has been identified, but instead a number of sperm proteins have been obtained, an effective immunoconceptive vaccine would be probably consisting of several specific antigenic epitopes (eliciting sperm-specific antibody response but lacking any side effects) included in a single formula. Perhaps, monospecific antibodies to sperm antigens may be combined for immunoconceptive purposes in the form of intravaginal sperm-specific spermicides. A first recombinant mini-antibody has been already engineered to the tissue-specific carbohydrate epitope located on the sperm glycoform of the CD52 [51] antigen and shown to agglutinate human sperm cells in a tangled pattern [52].

The identification of sperm antigens relevant to fertilization opens up opportunities also for diagnosis and treatment of immune infertility. At present, antisperm antibodies are suppressed systemically and direct targeting ASA is not possible. It may be speculated whether using monoclonal antibodies for treatment of immune infertility would be possible in the future.

List of abbreviations

ASA – antisperm antibodies

ECL – enhanced chemiluminescence

Mw – molecular weight

DIG – digoxigenin

ZP – zona pellucida

SP – seminal plasma

IEF – isoelectric focusing

NEPHGE – non-equilibrium pH gradient electrophoresis

2-D – two dimensional

SI – sperm immobilizing

IBT – immunobead binding test

MAR – mixed antiglobulin reaction

MALDI-MS – matrix assisted laser desorption ionisation mass spectrometry

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